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(54) Expression of HTLV-III gag-Gene.

(57) A recombinant gag-protein of the etiologic agent of acquired immune deficiency syndrome (AIDS) and the proteolytic proteins produced therefrom as well as corresponding vectors and transformants expressing these proteins are disclosed. In addition, a method of testing human blood for presence of antibodies to the AIDS virus using the recombinant gag-protein or any of its proteolytic proteins or mixtures thereof is disclosed.

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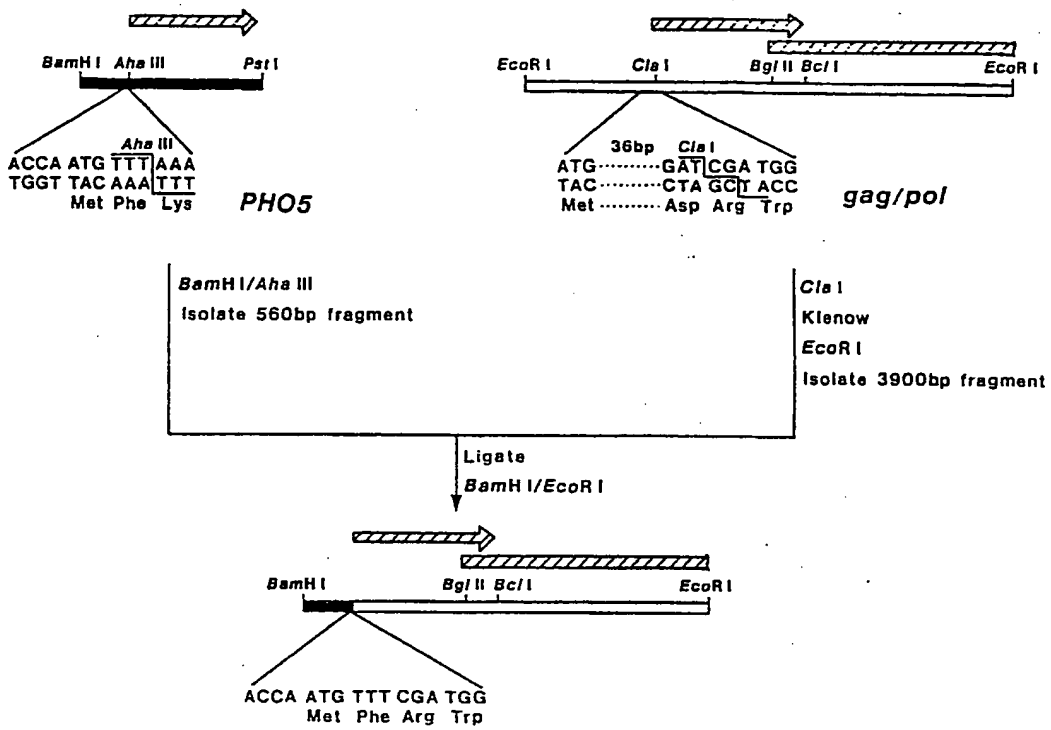

BamH I
Aha III
Pst I
Aha III
ACCA ATG TTT AAA
TGGT TAC AAA TTT
Met Phe Lys
PHO5
EcoR I
Cla I
Bgl II Bcl I
EcoR I
36bp
Cla I
ATG·····GAT CGA TGG
TAC·····CTA GCT ACC
Met·····Asp Arg Trp
gag/pol
BamH I/Aha III
Isolate 560bp fragment
Cla I
Klenow
EcoR I
Isolate 3900bp fragment
Ligate
BamH I/EcoR I
BamH I
Bgl II Bcl I
EcoR I
ACCA ATG TTT CGA TGG
Met Phe Arg Trp

Figure 1-B

Expression of HTLV-III gag-Gene

Background of the Invention

The retrovirus HTLV-III and the closely related variants of this virus. LAV and ARV, appear to be the causative agents of the disease Acquired Immunodeficiency Syndrome (AIDS) [see Barré-Sinoussi et al., Science 220, 868-871 (1983); Levy et al., Science 225, 840-842 (1984); Montagnier et al., in Human T-Cell Leukemia/Lymphoma Virus, R. C. Gallo, M. Essex and L. Gross, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory) pp. 363-370 (1984); Popovic et al., Science 224 . 497-500 (1984): Gallo et al., Science, 224 500-503 (1984); Schüpbach et al., Science 224, 503-505 - (1984)]. There is strong correlation between AIDS and the presence of antibodies to HTLV-III. Furthermore, 85-95% of patients with lymphadenopathy syndrome and a significant proportion of asymptomatic homosexual men in AIDS endemic areas carry circulating antibodies to HTLV-III [see Schüpbach et al., supra]. HTLV-III antibodies have also been widely detected in patients who were exposed to this disease through intravenous drug injections with contaminated needles and in hemophiliacs who received intravenous blood products. Current estimates indicate that approximately one million Americans have been infected with this virus, and approximately 10% of this infected population is expected to acquire this lethal disease.

Molecular cloning and nucleotide sequence analysis of HTLV-III and its variants have demonstrated that this viral genome exhibits many of the structural features of the avian and mammalian retroviruses [see Ratner et al., Nature 313, 277-284 (1985): Sanchez-Pescador et al., Science 227, 484-492 (1985); Wain-Hobson, et al., Cell 40, 9-17 (1985); and Muesing, M. et al., Nature 313, 450-458 (1985)]. Thus, the viral genome contains the three genes - (gag, pol and env) characteristic of all retroviruses. In addition, the HTLV-III genome contains two short open reading frames whose function are unknown.

Effective containment of AIDS depends on development of sensitive and rapid methods to identify individuals exposed to or infected with HTLV-III and therapeutic agents that interfere with viral replication. One of the viral genes, gag, encodes a precursor which is proteolytically processed into core proteins during virion maturation. From DNA sequence data and analysis of isolated viral proteins it follows that the HTLV-III gag precursor comprises about 56 kd and is processed into species of approximately 24, 16, and 14 kd (Ratner et al., supra; Sanchez-Pescador et al., supra; Wain-Hobson et al., supra; and Muesing et al., supra). The protease responsible for this processing is typically encoded by the retroviral genome. It is included in the 3′ end of the gag gene in avian retroviruses and in the 5′ end of the pol gene in mammalian viruses [for review see Dickson et al., "Protein Biosynthesis and Assembly", in Molecular Biology of Tumor Viruses, R. A. Weiss, N. M. Teich, H. E. Varmus and J. M. Coffin, eds. (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY) pp. 513-648 (1982)]. In at least one mammalian retrovirus, Moloney murine leukemia virus (MuLV), the protease is a gag-pol read-through product. A therapeutic agent that could inhibit this protease might block virus spread. It is, therefore, important to identify the region of the HTLV-III genome that encodes this protease and to develop an in vitro system in which the proteolysis of the gag gene precursor can be studied.

HTLV-III genomes have been molecularly cloned. Shaw et al., Science 226, 1165-1171 (1984). Also the complete nucleotide sequence of the proviral genome of HTLV III has been determined [Ratner et al., supra: and Sanchez-Pescador, et al., supra].

One reason for the difficulty in determining the etiologic agent of AIDS was due to the reactivity of various retroviral antigens with serum samples from AIDS Patients. For example, serum samples from AIDS patients have been shown to react with antigens of HTLV I and HTLV III (HTLV-I: Essex et al., "Antibodies to Cell Membrane Antigens Associated with Human T-Cell Leukemia Virus in Patients with AIDS", Science 220, 859-862 (1983); HTLV-III: Samgadharan et al., "Antibodies Reactive With Human T-Lymphotropic Retroviruses (HTLV-III) in the Serum of Patients With AIDS", Science 224, 506-508 (1984)). Gene products of HTLV demonstrated antigenicities cross-reactive with antibodies in sera from adult T-cell leukemia patients [Kiyokawa, T. et al., "Envelope proteins of human T-cell leukemia virus: Expression in Escherichia coli and its application to studies of env gene functions", PNAS - (USA) 81, 6202-6206 (1984)]. Adult T-cell leukemias (ATL) differ from acquired immune deficiency syndrome (AIDS) in that HTLV-I causes T-cell malignancies, that is uncontrolled growth of T-cell. In AIDS rather than cell growth there is cell death. In fact this cytopathic characteristic of HTLV III was critical to determining ultimately the specific retroviral origin of the disease. Thus the etiologic agent of AIDS was isolated by use of immortalized human neoplastic T-cell lines (HT) infected with the cytopathic retrovirus characteristic of AIDS, isolated from AIDS afflicted patients. Seroepidemiological assays using this virus showed a complete correla-

tion between AIDS and the presence of antibodies to HTLV III antigens [Samgadharan et al., supra - (1984); Schüpbach et al., supra]. In addition, nearly 85% of patients with lymphadenopathy syndrome and a significant proportion of asymptomatic homosexual men in AIDS endemic areas were also found to carry circulating antibodies to HTLV III. Taken together, all these data indicate HTLV III to be the etiologic agent for AIDS.

Until the successful culturing of AIDS virus using H-9 cell line the gag AIDS protein of the AIDS virus had not been isolated, characterized or synthesized. This in major part is due to the fact that the virus is cytopathic and thus isolation of the virus was not possible [Popovic, M. et al., supra]. Once the human T-cell line resistant to the cytopathic effects of the virus was discovered, a molecular clone of proviral DNA could be achieved.

The need for a sensitive and rapid method for the diagnosis of AIDS in human blood and its prevention by vaccination is very great. Virtually all the assays/tests presently available are fraught with errors. In fact the Center for Disease Control - (CDC) has indicated that presently available tests be used solely for screening units of blood for antibody to HTLV III. The CDC went further by stating that the presently available ELISA tests can not be used for general screening of high risk populations or as a diagnostic test for AIDS [Federal Register 50(48), 9909, March 12, 1985]. The errors have been traced to the failure to use a specific antigenic protein of the etiologic agent for AIDS. The previously used proteins were derived from a viral lysate. Since the lysate is made from human cells infected with the virus, i.e. the cells used to grow the virus, the lysate will contain human proteins as well as viral proteins. Thus preparation of a pure antigen of viral protein is very difficult. The antigen used produced both false positive and false negative results [Budiansky. S., AIDS Screening, False Test Results Raise Doubts, Nature 312. 583(1984)]. The errors caused by the use of such lysate proteins/peptides can be avoided by using a composition for binding AIDS antibodies which is substantially free of the non-AIDS specific proteins. Compositions that are substantially pure AIDS gag-proteins can be used as antigens.

Summary of Invention

In accordance with this invention we have produced by recombinant technology the immunologically active portion of the precursor of the HTLV-III gag protein as well as the immunological active portion of the natural proteolytic precusor proteins which result from this gag protein - (hereinafter also referred to as polypeptides immunologically equivalent to the gag-protein products of HTLV-III). In addition, we have produced a recombinant organism capable of expressing these proteins/polypeptides by utilizing various expression vectors capable of expressing all of these proteins. By recombinant technology in accordance with this invention, one produces the precursor gag 56 kd protein with modification resulting from the removal of N-terminal codons at its amino terminus, but having the same immunological activity as the natural precursor gag protein. In view of this modification in the precusor, the 14 kd gag protein which is proteolytically produced therefrom is also modified at its amino terminus from the natural 14 kd gag protein. However, this modified gag protein also has the same immunological activity as its natural form. Also in accordance with this invention, a new p48 protein is produced having the same immunological activity of the natural precursor gag protein. The 16 kd proteolytic protein produced by the process of this invention has a varient in its amino acid structure from the proteolytic 16 kd gag proteins reported in Shaw et al., supra. The 24 kd proteolytic protein is the same as the naturally occuring 24 kd proteolytic protein.

The 56 kd, 24, 16 and 14 kd gag proteins produced by this invention have the same immunological activity as their corresponding natural gag proteins. They have the same epitopes to react with the same antibodies as their corresponding natural proteins.

In accordance with this invention, recombinant DNA techniques are utilized for producing the HTLV-III gag protein having 56 kd as well as the proteolytic proteins produced therefrom, i.e. the proteins having 24,16 and 14 kd, respectively. In the first step of this invention, one isolates from the known genome for the retrovirus HTLV-III a portion which contains the DNA sequence encoding for the gag protein of HTLV-III and this portion is constructed into a gene which contains this DNA sequence encoding for the gag protein of HLTV-III operably linked to a promotor capable of effecting the expression of said DNA sequence and this gene is inserted into an expression vector or plasmid and such vector or plasmid is inserted into a suitable microorganism, preferably a yeast cell, to produce a microorganism capable of expressing the active portion of the gag-protein of HTLV-III. In accordance with this invention, the recombinant organism not only expresses a protein which is immunological equivalent to the natural precusor gag protein, but also expresses the species proteins which are processed by proteolytic enzymes expressed with the precusor protein.

Also included in this invention are those amino acid substitution in the sequence of the 56, 48, 24, 16 and 14 kd gag proteins produced through mutation of the recombinant microorganisms of this invention. These amino acids substitutions in the sequence of these proteins produce modified proteins which are immunologically equivalent to the proteins of which they are modifications. These amino acid substitutions are described in the art - [H. Neurath and R.L. Hill "The Proteins", Academic Press, New York (1979)] in particular in fig. 6 of page 14. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, and vice versa.

A further aspect of this invention relates to a diagnostic method for testing human blood for the presence of antibodies to the gag protein and its proteolytic proteins. This aspect of the invention overcomes the problems of previously used blood tests for AIDS. One of the problems in detecting in vitro the AIDS virus is to provide a composition which does not contain proteins or peptides which are not derived solely from the AIDS etiologic agent. A composition using either the active portion of gag-protein or its various proteolytically derived proteins overcomes the nonspecificity of the prior tests or assays. Yet another aspect of this invention is a diagnostic method for detecting and/or determining the presence of the antigen in human blood.

Another aspect of this invention is to use either the recombinant gag-protein or its proteolytically derived proteins as antigens in providing antibodies which are active in detecting AIDS in samples of body fluid.

Just another aspect of this invention is to use either the recombinant gag-protein or its proteolytically derived proteins as a vaccine capable of inducing protective immunity against the AIDS virus. Routes of administration, antigen doses, number and frequency of injections will vary from individual to individual and may parallel those currently being used in providing immunity in other viral infections. The vaccines can be prepared in accordance with known methods. The vaccine compositions will be conveniently combined with physiologically acceptable carrier materials. The vaccine compositions may contain adjuvants or any other enhancer of immune response. Furthermore, the vaccine compositions may comprise other antigens to provide immunity against other diseases in addition to AIDS.

The methods for testing human blood for the presence of AIDS virus or of antibodies against AIDS virus can be conducted in suitable test kits comprising in a container a recombinant gag-protein or its proteolytically derived proteins of the present invention or antibodies against AIDS virus elicited by these proteins of the present invention.

Brief Description of the Drawings.

Fig. 1-A illustrates the restriction sites in λ HXB-3, a known gene clone for the HTLV-III virus, with the gag region of this gene expanded to show the precursor (p 56) and its naturally proteolytically produced proteins (p 24, p 16, and p 14) as well as its mutant protein (p 48).

Fig. 1-B illustrates the construction of a gene containing the gag-gene for HTLV-III and a promoter for later insertion into a plasmid.

Fig. 2 is an immunoblot analysis of yeast lysates obtained from yeast grown with pYE72/gag 1. Column 1 is the reading taken from cells grown in a high phosphate medium and column 2 is the reading from cells grown in a phosphate-free medium. The indicated molecular weight markers indicate the sizes of the respective bands.

Figure 3 is an autoradiograph reading of an SDS gel at various times after immunoprecipitated lysates produced from yeast cells containing the gag plasmid pYE72/gag 1 grown with ^{35}S-methionine. Columns A through G in Fig. 3 represent different chase times. Column H represents results from lysates from yeast cells containing pYE 72/gag 1 grown with $^{32}PO_4$ and then harvested after 45 minutes and column I represents similar results as Column G except the plasmid inserted had no gag gene.

Fig 4 is an immunoblot analysis of yeast lysates produced through various mutations of the gag gene. The immunoblots were developed either with rabbit antibodies raised against disrupted HTLV-III (Part A of Fig. 4) or with AIDS patient serum (Part B of Fig. 4). In Parts A and B, column 1 is the result of lysates from cells induced with pYE72/gag 1, while column 2 is the results of the lysates from cells induced with pYE72/gag 2 and column 3 is the results of the lysate from cells induced with pYE72/gag 3.

Fig 5 is the DNA sequence of that portion the λ HXB-3 gene showing the gag/pol overlap. The carboxy-terminal coding region of gag and the amino-terminal coding region of pol are shown. The region that is homologous to other gag proteases - [Toh et al., Nature 315, 691 (1985)] is underlined. The reading frame to which the pol gene is shifted by the BclI fill in is shown by the arrow. periods i.e. "." in this figure indicate a translation termination site.

Fig. 6-A is an immunoblot analysis utilizing antigen produced by the lysates of yeast cells induced with pYE72/gag1, each of the columns re-

presents blood samples taken from a different AIDS patients living at the east coast of the United States.

Fig. 6-B is the same as Fig. 6-A except that the patients are taken from the west coast of the United States.

Fig. 7 is the DNA sequence encoding the 56 kd gag protein precusor produced in accordance with this invention.

Fig. 8 is the amino acid sequence of the 56 kd gag protein precusor produced in accordance with this invention.

Fig. 9 is the DNA sequence encoding the proteolytic 24 kd gag protein produced in accordance with this invention.

Fig. 10 is the amino acid sequence of the 24 kd proteolytic gag protein is produced in accordance with this invention.

Fig. 11 is the DNA sequence encoding the proteolytic 16 kd gag protein produced in accordance with this invention.

Fig. 12 is the amino acid sequence of the 16 kd proteolytic gag protein produced in accordance with this invention.

Fig. 13 is the DNA sequence encoding the 14 kd proteolytic gag protein produced in accordance with this invention.

Fig. 14 is the amino acid sequence of the 14 kd proteolytic gag protein produced in accordance with this invention.

Fig. 15 is the DNA sequence encoding the 48 kd proteolytic gag protein produced in accordance with this invention.

Fig. 16 is the amino acid sequence of the 48 kd proteolytic gag protein produced in accordance with this invention.

Detailed Description of the Invention

In the description the following terms are employed:

Nucleotide: A monomeric unit of DNA consisting of a sugar moiety (pentose), a phosphate, and either a purine or pyrimidine base (nitrogenous heterocyclic). The base is linked to the sugar moiety via the glycosidic carbon (1′ carbon of the pentose). That combination of a base and a sugar is called a nucleotide. Each nucleotide is characterized by its base. The four DNA bases are adenine - ("A"), guanine ("G"), cytosine ("C") and thymine - ("T").

DNA Sequence: A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3′ and 5′ carbons of adjacent pentoses.

Codon : A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"). TAG, TAA and TGA are translation stop signals and ATG is a translation start signal.

Reading Frame: The grouping of codons during translation of mRNA into amino acid sequences. During translation the proper reading frame must be maintained. For example, the sequence GCTGGTTGTAAG may be translated in three reading frames or phases, each of which affords a different amino acid sequence:

GCT GGT TGT AAG--Ala-Gly-Cys-Lys
G CTG GTT GTA AG--Leu-Val-Val
GC TGG TTG TAA G--Trp-Leu-(STOP)

Polypeptide : A linear array of amino acids connected one to the other by peptide bonds between the α-amino and carboxy groups of adjacent amino acids.

Genome: The entire DNA of a cell or a virus. It includes inter alia the structural genes coding for the polypeptides of the substance, as well as operator, promoter and ribosome binding and interaction sequences, including sequences such as the Shine-Dalgarno sequences.

Structural Gene : A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription: The process of producing mRNA from a structural gene.

Translation: The process of producing a polypeptide from mRNA.

Expression: The process undergone by a structural gene to produce a polypeptide. It is a combination of transciption and translation.

Plasmid : A circular double-stranded DNA Molecule that is not a part of the main chromosome of an organism containing genes that convey resistance to specific antibiotics. When the plasmid is placed within aunicellular organism, the characteristics of that organism may be changed or transformed as a result for the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

Cloning Vehicle : A plasmid, phage DNA or other DNA sequences which are able to replicate in a host cell, which are characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication,

production of coat proteins or loss of promoter or binding sites, and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

Cloning: The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA: A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

The nomenclature used to define the peptides or proteins is that used in accordance with conventional representation such that the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus to the right. By natural amino acid is meant one of common, naturally occurring amino acids found in proteins comprising Gly, Ala, Val, Leu, Ile, Ser, Thr, Lys, Arg, Asp, Asn, Glu, Gln, Cys, Met, Phe, Tyr, Pro, Trp and His. Where the amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented unless otherwise expressly indicated. In addition, amino acids have been designated by specific letters of the alphabet such that: A = Alanine; D = Aspartic Acid; N = Asparagine; C = Cysteine; D = Aspartic Acid; E = Glutamic Acid; F = Phenylalanine; G = Glycine; H = Histidine; I = Isoleucine; K = Lysine; L = Leucine; M = Methionine; N = Asparagine; P = Proline; Q = Glutamine; R = Arginine; S = Serine; T = threonine; V = Valine; W = Tryptophan; Y = Tyrosine; Q = Glutamine; E = Glutamic Acid.

In accordance with the present invention, the search for the protein of the etiologic agent for acquired immune deficiency syndrome (AIDS) has led to the isolation and sequencing of the proviral gene of the AIDS virus. It has now been discovered, for what is believed to be the first time that the postulated etiologic agents of AIDS, lymphadenopathy-associated virus (LAV), AIDS-Associated retrovirus (ARV) and human T-cell leukemia/lymphoma/lymphotropic virus (HTLV III) are in fact variants of the same virus. For purposes of this invention and claims the virus causing AIDS will be referred to herein as HTLV-III virus. HTLV-III virus will be understood to include the variants which have been postulated as the causative agent of AIDS, namely LAV and ARV .

As seen in Fig. I, the genome for HTLV-III is known, i.e. λHXB-3 and contains regions which code for the gag-protein, pol protein, sor-protein and envelope(env)-protein. The region of the genome which codes for the gag-protein is found within the 5.5 kb EcoRI fragment region and more particular within the Cla I through Bcl I region.

In accordance with this invention, in order to obtain the proteins of this invention, the HTLV-III gene is cut with one or more restriction enzymes to obtain the fragment which contains the gene encoding the gag-protein. This fragment is then ligated with a promoter to form a gene containing the promoter operably linked to a DNA sequence coding for the gag-protein. It is through this linking that modification at the amino terminus of the protein produced therefrom are introduced when expressed in an organism. In the next step the gene containing the promoter and DNA sequence encoding the gag-protein of HTLV-III is then inserted into a plasmid or expression vector replicable in a suitable microbiological host to form a plasmid or expression vector containing the promoter operably linked to the DNA sequence encoding the gag-protein for HTLV-III.

In the current state of the art, there are a number of promoter systems and suitable microbial hosts available which are appropriate to the present invention. Also, there are many types of plasmids into which the gene encoding the gag-protein of HTLV-III can be inserted. In general, plasmid expression vectors containing replication and controlled sequence, which are derived from species compatable with the host cell are used in connection with these hosts. For example, E. coli is typically transformed using plasmid pBR322, a plasmid derived from an E. coli species. For use with yeast, such as S. cerevisiae a plasmid such as pYE7 is generally utilized.

In accordance with this invention, any conventional promoter compatible with the host and the plasmid selected can be utilized. Promoters used for recombinant DNA construction in E. coli include the beta-lactamase (penicillinase) and lactose promoter such as disclosed by Chang et al., Nature 275; 615 (1978); Itakura et al., Science, 198; 1056 - (1977); promoter systems such as disclosed by Andersen et al., Mol. Cel. Bol. 3, 562-569 (1983) and Tryptophan promoter systems such as disclosed by Goeddel et al., Nucleic acids Res. 8, 4057 (1980) also EPO Appl. No. 0036776. In yeast, promoters including but being not limited to those from ADC1, GAL1, GAL10, PHO5, PGK1 and GAP1 have been used as reviewed by Broach et al. - (1983) in Experimental Manipulation of Gene Expression, M. Inouye, ed., Academic press: New York, N.Y., pp 83-117. While these are the most commonly used, other microbial promoters have

been discovered and utilized and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally in an operable relationship to the genes in transformation vectors [Sibenlist et al., Cell 20, 269 (1980)].

A wide variety of host/cloning vehicle combinations may be employed in cloning the double-stranded DNA. For example, useful cloning vehicles may consist of segments of chromosomal, nonchromosomal and synthetic DNA sequences, such as various known bacterial plasmids, e.g., plasmids from E. coli such as pBR322, phage DNA, and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids. Useful hosts may include microorganisms, mammalian cells, plant cells and the like. Among them microorganisms and mammalian cells are preferably employed. As preferable microorganisms, there may be mentioned yeast such as S. cerevisiae and bacteria such as Escherichia coli, Bacillus subtillis, Bacillus stearothermophilus and Actinomyces. The above-mentioned vectors and hosts may also be employed for the production of a protein from a gene obtained biologically as in the instant invention. Of course, not all host/vector combinations may be equally efficient. The particular selection of host/cloning vehicle combination may be made by those skilled in the art after due consideration of the principles set forth without departing from the scope of this invention.

Furthermore, within each specific cloning vehicle, various sites may be selected for insertion of the double-stranded DNA. These sites are usually designated by the restriction endonuclease which cuts them. For example, in pBR322, the EcoRI site is located just outside the gene coding for ampicillin resistance. Various sites have been employed by others in their recombinant synthetic schemes. Several sites are well recognized by those of skill in the art. It is, of course, to be understood that a cloning vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vehicle could be joined to the fragment by alternative means.

The vector or cloning vehicle and in particular the site chosen therein for attachment of a selected DNA fragment to form a recombinant DNA molecule is determined by a variety of factors, e.g. number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination of the protein to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a particular gene is determined by a balance of these factors, not all selections being equally effective for a given case.

There are several known methods of inserting DNA sequences into cloning vehicles to form recombinant DNA molecules which are equally useful in this invention. These include, for example, direct ligation, synthetic linkers, exonuclease and polymerase-linked repair reactions followed by ligation, or extension of the DNA strand with DNA polymerase and an appropriate single stranded template followed by ligation.

It should, of course, be understood that the nucleotide sequences of the DNA fragment inserted at the selected site of the cloning vehicle may include nucleotides which are not part of the actual structural gene for the desired polypeptide/protein or may include only a fragment of the complete structural gene for the desired protein. It is only required that whatever DNA sequence is inserted, a transformed host will produce a protein/peptide having an immunological activity to the AIDS gag-protein or that the DNA sequence itself is of use as a hybridization probe to select clones which contain DNA sequences useful in the production of polypeptides/proteins having an immunological activity to the AIDS gag-protein.

The cloning vehicle or vector containing the foreign gene is employed to transform a host so as to permit that host to express the protein or portion thereof for which the hybrid DNA codes. The selection of an appropriate host is also controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for expression of a particular recombinant DNA molecule.

Once the organism capable of carrying out the expression of the gag gene has been created, the process of this invention can be carried out in a variety of ways depending upon the nature of the construction of the expression vectors for the gag gene and upon the growth characteristics of the host. Typically, the host organism will be grown under conditions which are favorable to production of a large quantities of cells. When a large number of cells has accumulated suitable inducers or derepressors in the growth medium cause the promoter supplied with such gene sequence to become active permitting the transcription and trans-

lation of the coding sequence. The protein produced by the recombinant cell can be lysated by conventional means well known in the art. It is apparent that the particular means of lysating will depend upon the host cell utilized.

In a preferred embodiment of this invention, the HTLV-III gag gene is introduced into a yeast expression vector with a promoter which is not activated in a phosphate medium. This promoter was obtained from PHO5 as described by Thill et al., Mol. Cell Biol. 3, 570-579 (1983). The gag gene was obtained from a 5.5Kb Eco RI fragment from the HTLV-III clone λ HXB-3 [Shaw et al. Science 226, 1165-1171 (1984)]. This is the Eco RI fragment of the HTLV-III genome illustrated in Fig. 1-A.

Fig. 1-B illustrates the formation of the hybrid gene containing the PHO5 promoter and the gag gene fragment in Fig. 1-A. The yeast promoter and translation initiation site are on a 560bp BamHI to AhaIII restriction fragment derived from the gene for repressible acid phosphatase, PHO5. The enzyme AhaIII produces a blunt end just after the second codon of PHO5 as seen in Fig. 1-B. The gag gene within the 5.5 kd EcoRI fragment is ligated to the promoter obtained from the BamHI to AhaIII restriction fragment derived from PHO5. The EcoRI fragment from the HTLV-III clone contains the entire gag gene and a large part of the pol gene which overlaps the gag gene in a different reading frame (Ratner et al., supra; Sanchez-Pescador et al., supra; Wain-Hobson et al., supra; and Muesing et al., supra). The EcoRI fragment was cut with ClaI near the amino terminus of the gag gene and the resulting 5' overlap was filled in with DNA polymerase large fragment to create a blunt-ended DNA molecule beginning with an arginine codon. Ligation of this end to the AhaIII of the PHO5 fragment fused the promoter and the first two codons of PHO5 to the fifteenth codon of the gag gene.

The fused gene prepared above was then inserted into a pYE7 vector, a vector that can both replicate in yeast and E. coli. This recombinant plasmid was labelled pYE72/gagI. The resulting plasmid was then used to transform yeast.

In carrying out this ligation, any conventional method of ligation can be utilized to fuse the promoter to the gag gene. Any conventional method of transforming a microorganism such as yeast with a plasmid can be utilized to produce the recombinant organism which will express the gag gene.

The PHO5 promoter in the plasmid labelled pYE72/gagI was induced in the yeast cells by growth in a phosphate-free medium and extracts of the cells were analyzed for the present of the gag-specific proteins by immunoblot analysis using rabbit polyclonal antiserum to disrupted virus as shown in Fig. 2. The column labelled 1, in Fig. 2, represents the results from the cells with pYE72/gagI grown in a high phosphate containing medium whereas column 2 represents the results from cells with pYE72/gagI grown in a phosphate-free medium. The indicated molecular weights are used to determine the sizes of the reactive band. As seen from Fig. 2, there was no expression of the gag gene in the phosphate containing medium were the promoter was not activated to produce the gag gene. On the other hand, when the same cells were grown in a phosphate-free medium, the lysates produced immuno reactive proteins which correspond to the gag protein and its various known proteolytic proteins which are formed therefrom.

As seen from column 2 in Fig. 2, a major immunoreactive protein produced corresponded in size to the HTLV-III p24 gag protein identified in virions and predicted from the known DNA sequence. The reactive species of the sizes expected for the p14 and p16 proteins were detected as well. A larger protein of about 56 kd size which corresponded to the entire gag protein as well as several species of about 40 kd which represents a proteolytic processing intermediate were also produced.

To determine whether the recombinant HTLV-III gag protein is actually processed by proteolysis in yeast to produce the actual viral specific proteolytic proteins, the proteins obtained from the lysate of the yeast transformed with pYE72/gagI was followed in a pulse chase experiment with S^{35}-methione or $^{32}PO_4$. Fig. 3 shows the results of this experiment, each of the columns represent a different chase time as follows: A) 0 min, B) 2 min, C) 5 min, D) 10 min, E) 20 min, F) 30 min, G) 45 min. In all respects the complete gag gene of 56 kd was detected with radioactivity first. The protein of about 25 kd seen in Fig. 2 was also detected before the 24 Kd protein and thus may be its immediate precursor. Protein migrating as expected of 16 Kd was also detected. The p14 region of the gag gene has no methionine. Therefore, the failure to detect this protein supports its proposed origin.

Phosphorylation of the recombinant gag proteins made in yeast was examined after growth of the yeast cells transformed with pYE72/gagI in a low phosphate medium containing $^{32}PO_4$. In Fig. 3 columns H and I show the results of gel analysis of labelled proteins immunopercipitated with rabbit HTLV-III antibodies. In column H where the transformed yeast was utilized, the 56 kd, one of the 40 kd intermediates, the 25 kd, the 24 kd and the 16 kd species were all radioactive. No radioactivity was seen at the position of the 14 kd band when a

higher percentage of gel was run. Thus the p24 and p16 gag protein appear to be phosphorylated in yeast. No gag proteins were seen when the non-transformed yeast was utilized as in column I.

As seen from Fig. I-A presence of a BglII restriction site near the carboxy-terminal coding region of the gag gene provides a means to eliminate about half of the p16 coding region as well as most of the pol gene present in 5.5 kd EcoRI fragment. The carboxy-terminal part of the gag polyprotein and/or the amino terminal region of the pol gene has been shown to encode a processing protease in many retroviruses (see, for example, Dickson et al., supra).

In order to remove the protease from the recombinant gag protein the BglII to EcoRI portion of the EcoRI fragment of HTLV-III genome was removed (see Fig. I-B). It is believed that the removed portion of the gag gene encodes proteases which convert the large recombinant gag protein into its various mature protein species. Therefore, the BamHI-EcoRI gene prepared in Fig. I-B can be fragmented by treating either the gene or the plasmid with BglII so as to remove the BglII to EcoRI fragment from this gene. If the expression plasmid pYE72/gagI is used, this plasmid is opened by digesting with BamHI and BglII. Once the BamHI to BglII portion of this EcoRI fragment is obtained, this portion may be inserted into a suitable plasmid, depending upon the microorganism into which it is to be grown. In inserting this portion of the gene into a plasmid it may be necessary to ligate this portion to series of DNA bases which permit insertion into the desired plasmid. In the case where pYE7 is utilized, the BamHI to BglII fragment is ligated with the 375 bp BamHI-EcoRI fragment from pBR322 to form a BamHI to EcoRI fragment which can be inserted into pYE7 to form pYE72/gag2.

In accordance with another embodiment of this invention another mutation of the gag gene was introduced at the BclI restriction site in the pol gene just downstream of the gag, i.e. pYE72/gag3. This mutation involves introducing four base pairs at the BclI site which cause a frame shift out of the pol reading frame as shown in Fig. 5. In Fig. 5 the carboxy terminal coding region of the gag gene and the amino terminal coding region of the pol gene are shown. The region that is homologous to other gag proteases is underlined in this figure. The reading frame created by the BclI fill in is shown by the arrow. A period indicates a translational terminal site. The frame shift caused by the insertion of the bases deactivated the gene from producing certain proteases which may proteolytically cleave the gag protein.

The products formed by the full recombinant gag gene and the truncated gag genes are shown in figure 4. Column I in Fig. 4 represent immunoblots taken from lysates of cells induced with pYE72/gagI. Column 2 in Fig. 4 is directed to the result of immunoblots taken from lysates from cells induced with pYE72/gag2; and column III represents immunoblots taken from lysates from cells induced with pYE72/gag3. As seen, pYE72/gagI and 3 produced the large protein p56. For pYE72/gag3, the major immunoreactive protein band was about 56 kd and comigrated with the presumptive precursor protein seen in the cells with induced pYE72/gagI. However, the mutation also appears to prevent processing of this recombinant gag precursor. As with the deletion mutant, a smaller amount of p24 appeared to be present as compared with protein seen in the cells induced with pYE72/gagI. In addition to a major 56 kd product and the smaller species, a band at about 60 kd was detected in the frame shift mutant. This size would be consistent with gag/pol fusion product that was terminated as shown in Fig. 5 as a result of the BclI filling in.

Screening of AIDS SERA

Because anti-HTLV-III antibodies are found in more than 90% of the AIDS patients, the microbially synthesized gag gene products can be used as diagnostic tools for the detection of these antibodies. For this analysis as seen in Figures 6-A and 6-B, total cell protein from the yeast culture induced with pYE72/gagI was fractionated by SDS-PAGE and transferred to a nitrocellulose filter by Western blotting technique. Strips of the filter containing transferred proteins were reacted with 1000-fold diluted human sera, and the antigen-antibody complexes formed were detected by incubation of the strips with 125-I-labelled Staphylococous aureus protein A followed by autoradiography. Prominent bands corresponding to reaction of the antibody to the 56 kd, 24 kd, 16 kd and 14 kd proteins were consistently observed when the serum used was from patients with AIDS syndrome. The results of one such assay with 11 human sera from patients on the East cost are presented in Figure 6-A. Similar results with 11 serum samples from west cost patients appear in Fig. 6-B. The negative controls (not shown) used were normal human sera. No reaction observed with sera from healthy individuals.

It appears, therefore, that the recombinant gag gene products can be used as diagnostic reagents for the detection of AIDS associated antibodies. The recombinant gag gene products of the instant invention encompasses a large portion of the protein molecule and contains both the conserved and

divergent portions of the molecule. In spite of the divergence observed between HTLV-III and ARV-2 sequences the recombinant gag protein products of the instant invention synthesized by the bacteria react with AIDS patient sera derived from both geographical locations of the United States. One hundred percent (100%) of AIDS patient sera (22 individual samples, 11 derived from the East Coast of the United States and 11 derived from California) tested showed high reactivity. This is strong evidence for the presence of conserved epitopes within the molecule against which the immune system could mount an antibody reaction. The human immune system may thus be mounting an immune response against conserved epitopes of the gag proteins molecule by the reactivity of the AIDS patient sera.

Based on these discoveries it is proposed that in the practice of screening blood for Acquired Immune Deficiency Syndrome (AIDS), the AIDS recombinant gag protein products of this invention can be utilized. Utilizing the protein products of the instant invention, human blood can be screened for the presence of antibodies to the AIDS virus. This and other techniques are readily determined. The foregoing and other objects, features and advantages of the invention will be apparent from the following examples of preferred embodiments of the invention.

In the Examples, E. coli strain MC1061 was the same as described by Casadaban et al., J. Mol. Biol. 138, 179-207 (1980). The E. coli strain GM119 used for the preparation of the unmethylated DNA, was that described by Arraj et al., J. Bact. 153, 562-563 (1983). E. coli strains MC 1061 and GM 119 were deposited at American Type Culture Collection (ATCC) on November 26, 1985 the accession nos. being ATCC 53338 and ATCC 53339, respectively. The yeast expression plasmid pYE7 used in Example 5 was the same as described in Examples and Fig. 6 of European Patent Application publication No. 0124824 which is incorporated by reference. The yeast used was S. cerevisiae 20B-12 (ATCC No. 20626). Yeast transformation was performed as described by Hinnen et al., Proc. Nat. Acad. Sci.. USA, 75, 1929-1934 (1978). The PHO5 gene utilized to produce the promoter was obtained from the plasmid pAP20 as described by Andersen et al., Mol. Cell Biol. 3 562-569 (1983). The λ HXB-3 used, was as described by Shaw et al., Science 226, 1165-1171 (1984).

Example 1

Preparation of pYE72/gag1

Restriction and DNA modifying enzymes were used as recommended by the supplier. All restriction enzyme digests were performed at 37°C for 1 hr with 0.5-1.0 units of enzyme in 50mM NaCl, 10mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 1mM dithiothreitol (DTT). The 560 bp BamHI to AhaIII fragment with PHO5 promoter and translation initiation region was obtained from the plasmid pAP20, and the ClaI to EcoRI fragment with the gag/pol region was obtained from λ HXB-3. The 5.5 kb. EcoI fragment was subcloned into pBR3222 and the plasmid grown in E. coli GM119. The ClaI 5′ overhang of the ClaI to EcoRI-fragment was "filled in" by treatment with 5 units of the Klenow fragment of E. coli DNA polymerase I in the presence of all four deoxyribonucleotides at 50μM (C,T,A, and G) at 16°C for 2 hr. in the same buffer as that used for restriction enzyme reactions.

Approximately equal amounts of the BamHI-AhaIII PHO5 fragment and the ClaI (filled-in)-EcoRI gag/pol fragment were treated with 1.0 units of T4 DNA ligase for 16 hr at 16°C in 50mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 20mM DTT, 1mM αATP. The products were cut with BamHI and EcoRI and the PHO5-gag/pol fusion was inserted by ligation into pYE7 which had been cut with BamHI and EcoRI. The resulting expression plasmid was designated pYE72/gag1.

Example 2

Preparation of pYE72/gag2

A deletion that removed the carboxy-terminal portion of gag and all of pol was made by digesting pYE72/gag1 with BamHI and BglII and isolating the PHO5-gag fragment. This was converted to a BamHI to EcoRI fragment by ligation with the 375 bp BamHI-EcoRI fragment from pBR322 to the BglII site through the identical 5′ overhangs of BglII and BamHI. Insertion of this fragment into pYE7 yielded PYE72/gag2.

Example 3

Preparation of pYE72/gag3

Another mutation was introduced at the BclI site in the pol gene just downstream of gag. Since BclI does not cut methylated DNA, pYE72/gag1 was introduced into E. coli GM119, and DNA was prepared and cut with BclI. The 5′ overhang was filled in with Klenow fragment as above and the plasmid

was recircularized by blunt-end ligation. The resulting plasmid with the 4 bp GATC insert at the BclI site was designated pYE72/gag3.

Example 4

Growth and induction of Yeast Strains

Separate cultures of the yeast strain S. cerevisiae 20B-12 containing the gag expression plasmids or a vector with no inserted gene were grown in the medium YCAD and induced in phosphate-free medium as described by Kramer et al., Proc. Natl. Acad. Sci USA 81, 367-370 (1984). About 6 hr after transfer to phosphate-free medium, cells were collected by centrifugation and spheroplasts prepared with Zymolyase 60,000 - [Kaneko, T., Kitamura, K., and Yamamoto, Y., Agr. Biol. Chem., 37, 2295 (1973)] and collected. spheroplasts were usually stored at -20°C before analysis. For protein purification, whole cells instead of spheroplasts are collected and frozen.

Example 5

Purification of the recombinant gag Protein Products

A homogeneous recombinant gag protein or its proteolytic products can be purified according to the following procedure. The induced yeast cells are broken by standard mechanical procedures. These include passage through a French Pressure Cell or rapid mixing with glass beads in a mixer such as a Bead Beater, a Dyna-Mill or a Braun Homogenizer. For any of the above, the cell paste is resuspended in approximately 2 volumes - (relative to cell pellet) of 50mM $NaPO_4$, pH7.4 buffer. For glass bead lysis, an equal volume of 0.5 mm glass beads is added. Lysis is accomplished by either three passages at 20,000 p.s.i. through the French Pressure Cell or as recommended by the manufacturer of the mixer. Lysis can be monitored by microscopy.

Following cell breakage, cell debris (and glass beads) are removed by two 10 min centrifugation at 600 x g. Another centrifugation at 12,000 x g for 20 min removes mitochondria. The proteins are then fractionated by centrifugation at 100,000 x g for 1 hr to obtain a pellet (microsomal fraction) and supernatant (soluble fraction). If the gag proteins are in the microsomal fraction, solubilization using either various ionic and/or non-ionic detergents or denaturing reagents such as urea or guanidine HCl is necessary prior to further purification.

Additional purification can be achieved by standard liquid chromatography procedures. Different chromatography media can be used to obtain fractionation by gel filtration, ion exchange chromatography, and/or affinity chromatography. For gag proteins, single-stranded DNA cellulose affinity chromatography should be useful since gag proteins bind to nucleic acids.

Final purification can be obtained by reverse phase high performance liquid chromatography - (HPLC). The HPLC step yields the precursor gag protein and the natural proteolysis proteins derived therefrom in a substantially 100% pure form. It is also foreseeable that monoclonal antibody affinity chromatography columns utilizing gag polyclonal or monoclonal antibodies to the precursor gag protein and the natural proteolysis proteins, could be used as an alternative to HPLC.

By the above purification procedure, one obtains the following products:

The 56 kd protein having the structure given in Fig 8;

The 24 kd protein having the structure given in Fig 10;

The 16 kd protein having the structure given in Fig 12;

The 14 kd protein having the structure given in Fig 14;

and

The 48 kd protein having the structure given in Fig 16.

Example 6

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

For the immunoblot analysis of Figs. 2, 3, 4, 6-A and 6-B, cells were lysed by resuspending the spheroplast pellets (approximately 10^8 cells) in an equal volume of 2 x sample buffer of Laemmli - (Laemmli, "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4", Nature 227, 68-685, 1970) and incubated at 95°C for five (5) minutes. Debris were pelleted by centrifugation and the cleared lysates were subjected to SDS-PAGE analysis, id. For Western blot analysis, the proteins from the acrylamide gel were electroblotted onto a 0.1 micrometers nitrocellulose membrane (Schleicher and Schuell) for 16 hr at 50V, in 12.5 mM Tris, 96 mM glycine, 20% methanol, 0.01% SDS at pH 7.5. Processing of the blot was carried out using the methods described by Towbin et al., "Electrophoretic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose Sheet: Procedure and Some applications," Proc. Natl. Acad. Sci. U.S.A., 76, 4350-4354, (1979). For treat-

ment with the human sera, the blots were incubated with a 1000 fold dilution of the sera in antibody buffer (20 mM sodium phosphate buffer, pH 7.5 containing 0.5 M NaCl, 1% BSA and 0.05% Tween 20) for 2-6 hr. The blots were then washed twice with phosphate buffered saline containing 0.05% Tween 20 and then incubated with 125-I-labelled Staphylococous aureus protein A for an additional period of 1 hr. The blot was then washed twice in PBS-Tween 20 buffer, dried and autoradiographed.

Example 7

Labelling of the Cells

The cells were labelled with either ^{35}S-methionine pulse-chase or $^{32}PO_4$ for the pulse chase immunopre cipitation of Fig. 3 by the following procedure. For the ^{35}S-methionine pulse-chase, 500 mCi ^{35}S-methionine was added to the culture medium. After a 2 min labelling period, unlabelled methionine was added. At various times,the cells were harvested, lysed and processed by immunoprecipitation with rabbit antibody, and the lysates were analyzed by SDS-PAGE and autoradiography. The chase times are as follows: A) 0 min, B) 2 min, C) 5 min, D) 10 min, E) 20 min, F) 30 min, G) 45 min. For the $^{35}PO_4$ labeling in Columns H and I, 1 mCi $^{32}PO_4$ was added to the culture medium. Labeling was continued for 30 min. at 30°C, then cells were harvested, and lysates processed for immunoprecipitation as described above. Lysates were from cells containing pYE72/gag1 (H), and cells containing a similar plasmid with no gag gene (I).

Example 8

Diagnostic Test for AIDS

It is clear that the recombinant precursor gag protein and the natural proteolysis proteins derived therefrom, of the instant invention may be used as diagnostic reagents for the detection of AIDS-Associated antibodies. It is also apparent to one of ordinary skill that a diagnostic assay for AIDS using polyclonal or monoclonal antibodies to the AIDS recombinant gag precursor protein or the proteolytic products may be used to detect the presence of the AIDS virus in human blood. In one embodiment a competition immunoassay is used where the antigenic substance, in this case the AIDS virus, in a blood sample competes with a known quantity of labelled antigen, in this case labelled AIDS recombinant precursor gag protein, or the proteolysis proteins derived therefrom for a limited quantity of antibody binding sites. Thus, the amount of labelled antigen bound to the antibody is inversely proportional to the amount of antigen in the sample. In another embodiment, an immunometric assay may be used wherein a labelled AIDS gag antibody which complexes with the antigen-bound antibody is directly proportional to the amount of antigen (AIDS virus) in the blood sample. In a simple yes/no assay to determine whether the AIDS virus is present in blood, the solid support is tested to detect the presence of labelled antibody. In another embodiment, monoclonal antibodies to recombinant precursor AIDS gag protein, or the natural proteolysis proteins derived therefrom, may be used in an immunometric assay. Such monoclonal antibodies may be obtained by methods well known in the art, particularly the process of Milstein and Kohler reported in Nature 256, 495-497 (1975). The antigens in this assay can be the recombinant gag precursor protein or the proteolysis proteins derived therein either in pure form or as a mixture of these proteins.

The immunometric assay method is as follows: Duplicate samples are run in which 100 µl of a suspension of antibody immobilized on agarose particles is mixed with 100 µl of serum and 100 µl of soluble ^{125}I-labelled antibody. This mixture is for specified times ranging from one-quarter hour to twenty-four hours. Following the incubation periods the agarose particles are washed by addition of buffer and then centrifuged. After removal of the washing liquid by aspiration, the resulting pellet of agarose particles is then counted for bound ^{125}I-labelled antibody. The counts obtained for each of the complexes can then be compared to controls.

Various features of the invention are set forth in the following claims.

Claims

1. A polypeptide immunologically equivalent to the gag-protein products of HTLV-III having the amino acid sequence given in either Fig. 8 or Fig. 16, or 14kd, 16kd or 24kd proteolytic polypeptide fragments thereof or polypeptides related to any of said polypeptides by amino acid substitution(s) which occur through mutations of a recombinant host cell which produces said polypeptides.

2. The polypeptide of claim 1 wherein said polypeptide is the 56kd precursor having the amino acid sequence:

MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-
NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-
TVATLVCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQA-
ISPRTLNAWVKVVEEK AFSPEVIPMFSALSEGATP-

QDLNTMLNTVGGHQAAMQMLKETINEEAAEW
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQI-
GWMTNNPPIPVGEIY KRWIILGLNKIVRMYSPTSIL-
DIRQGPKEPFRDYVDRFYKTLRAEQASQE
VKNWMTETLLVQNANPDCKTILKALGPAATLEEN-
MTACQGVGGPGHKARV LAEAMSQVTNTATIMM-
QRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG
CWKCGKEGHQMKDCTERQANFLGKIWPSYKGRP-
GNFLQSRPEPTAPPFLQ SRPEPTAPPEESLRSG-
VETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

3. The polypeptide of claim I wherein the polypeptide is a I4kd proteolytic fragment having the amino acid sequence:
MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-
NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-
TVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNY

4. The polypeptide of claim I wherein said polypeptide is the 48kd proteolytic fragment having the amino acid sequence:
MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-
NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-
TVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQA-
ISPRTLNAWVKVVEEK AFSPEVIPMFSALSEGATP-
QDLNTMLNTVGGHQAAMQMLKETINEEAAEW
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQI-
GWMTNNPPIPVGEIY KRWIILGLNKIVRMYSPTSIL-
DIRQGPKEPFRDYVDRFYKTLRAEQASQE
VKNWMTETLLVQNANPDCKTILKALGPAATLEEM-
MTACQGVGGPGHKARV LAEAMSQVTNTATIMM-
QRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG
CWKCGKEGHQMKDCTERQANFLGKIHRTGVVAM-
IA

5. The polypeptide of claim I wherein said polypeptide is the I6kd proteolytic fragment having the amino acid sequence:
MQRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKK-
GCWKCGKEGHQMKDCT ERQANFLGKIWPSYKG-
RPGNFLQSRPEPTAPPFLQSRPEPTAPPEESLRS
GVETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

6. The polypeptide of claim I wherein said polypeptide is the 24kd proteolytic fragment having the amino acid sequence:
PIVQNIQGQMVHQAISPRTLNAWVKVVEEKAFSPE-
VIPMFSALSEGATPQ DLNTMLNTVGGHQAAMQM-
LKETINEEAAEWDRVHPVHAGPIAPGQMREPR
GSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIIL-
GLNKIVRMYSPTSI LDIRQGPKEPFRDYVDRFYKT-
LRAEQASQEVKNWMTETLLVQNANPDCKT ILKAL-
GPAATLEEMMTACQGVGGPGHK

7. A polypeptide immunologically equivalent to the gag-protein products of HTLV-III expressed in yeast.

8. A gene containing a gene portion having a DNA sequence encoding a polypeptide as claimed in any one of claims I to 6 operably linked to a promoter capable of effecting the expression of said DNA sequence.

9. The gene of claim 8 wherein said gene portion is the ClaI to EcoRI restriction site fragment of the λ HXB-3 genome.

I0. The gene of claim 9 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

II. The gene of claim 8 wherein said gene portion is the ClaI to BglII restriction site fragment of the ClaI to EcoRI fragment of the λ HXB-3 genome.

I2. The gene of claim II wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

I3. The gene of claim 8 where the gene portion is the ClaI to EcoRI restriction site fragment of λ HXB-3, wherein the ClaI to EcoRI fragment is filled in at its BclI restriction site with the base sequence GATC.

I4. The gene of claim I3 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

I5. A gene containing a gene portion having a DNA sequence encoding a polypeptide immunologically equivalent to the gag-protein products of HTLV-III operably linked to a promoter capable of effecting the expression of said DNA sequence in yeast.

I6. A recombinant expression vector capable of effecting the expression of a polypeptide as claimed in any one of claims I to 6 containing a gene portion having a DNA sequence encoding said polypeptide operably linked to a promoter capable of effecting the expression of said DNA sequence.

I7. A vector according to claim I6 wherein the gene portion is the ClaI to EcoRI restriction fragment of the λ HXB-3 genome.

I8. A vector according to claim I7 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

I9. A vector according to claim I6 wherein the gene portion is the ClaI to BglII restriction site fragment of the Cla I to EcoRI restriction site fragment of the λ HXB-3 genome.

20. A vector according to claim I9 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

2I. A vector according to claim I6 wherein the gene portion is the ClaI to EcoRI restriction site fragment of λ HXB-3, wherein the ClaI to EcoRI fragment is filled in at the BclI restriction site with the base sequence GATC.

22. A vector according to claim 2l wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

23. A vector according to claim l8 which is pYE72/gagl.

24. A vector according to claim 20 which is pYE72/gag2.

25. A vector according to claim 22 which is pYE72/gag3.

26. A recombinant expression vector capable of effecting the expression of a polypeptide immunologically equivalent to the gag-protein products of HTLV-III in yeast containing a gene portion having a DNA sequence encoding the gag-protein of HTLV-III operably linked to a promoter capable of effecting the expression of said DNA sequence.

27. A transformed cell carrying a vector as claimed in any one of claims l6 to 25.

28. A transformed cell according to claim 27 which is a yeast cell.

29. A transformed cell according to claim 28 which is a S. cerevisiae yeast cell.

30. A transformed cell according to claim 29 which is S. cerevisiae 20B-l2.

3l. A transformed yeast cell carrying a vector as claimed in claim 26.

32. A polypeptide according to any one of claims l to 7 as constituent of a vaccine.

33. A polypeptide according to any one of claims l to 7 as antigen.

34. A process for producing a polypeptide as claimed in any one of claims l to 6 comprising: transforming a host cell with an expression vector as claimed in any one of claims l6 to 25;

culturing said host cell so that the protein products are expressed; and,

extracting and isolating said protein products.

35. A process according to claim 34 wherein said host cell is a yeast cell

36. A process according to claim 35 wherein said yeast cell is a S. cerevisiae cell.

37. A process for producing a polypeptide immunologically equivalent to the gag-protein products of HTLV-III comprising: transforming a yeast cell with an expression vector as claimed in claim 26;

culturing said yeast cell so that the protein products are expressed; and,

extracting and isolating said protein products.

38. A method of testing human blood for the presence of antibodies to the viral etiologic agent of AIDS which comprises mixing a composition containing a polypeptide as claimed in any one of claims l to 7 or mixtures thereof with a sample of human blood and determining whether said protein or any of its natural proteolytic proteins or mixtures thereof binds to AIDS antibodies present in the blood sample.

39. Vaccines containing a polypeptide as claimed in any one of claims l to 7 and a physiologically acceptable carrier.

40. Antibodies raised aqainst a polypeptid as claimed in any one of claims l to 7.

4l. The antibodies of claim 40 which are monoclonal antibodies.

42. The use of a polypeptide as claimed in any one of claims l to 7 for the preparation of a protective immunization vaccine.

43. The use of a polypeptide as claimed in any one of claims l to 7 for the preparation of antibodies against AIDS virus.

44. The use of a polypeptide as claimed in any one of claims l to 7 for testing human blood for the presence of AIDS virus.

45. A test kit for the determination of antibodies against AIDS virus comprising in a container a polypeptide according to any one of claims l to 7.

46. A test kit for the determination of AIDS virus comprising in a container antibodies against AIDS virus elicited by a polypeptide according to any one of claims l to 7.

Claims for the following Contracting States : AT; ES

l. A process for producing polypeptides immunologically equivalent to the gag-protein products of HTLV-III having the amino acid sequence given in Fig. 8 or Fig. l6, or l4kd, l6kd or 24kd proteolytic fragments thereof, which process comprises: transforming a host cell with an expression vector comprising a gene coding for said gag-protein products operably linked to a promoter sequence enabling transcription, translation and expression of said gag-protein products in said host cell;

culturing said host cell so that the protein products are expressed; and,

extracting and isolating said protein products.

2. A process according to claim l, characterized in that a host cell is transformed with an expression vector capable of expressing a polypeptide with the amino acid sequence:

MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-
NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-
TVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQA-
ISPRTLNAWVKVVEEK AFSPEVIPMFSALSEGATP-
QDLNTMLNTVGGHQAAMQMLKETINEEAAEW
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQI-
GWMTNNPPIPVGEIY KRWIILGLNKIVRMYSPTSIL-
DIRQGPKEPFRDYVDRFYKTLRAEQASQE
VKNWMTETLLVQNANPDCKTILKALGPAATLEEM-
MTACQGVGGPGHKARV LAEAMSQVTNTATIMM-
QRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG

CWKCGKEGHQMKDCTERQANFLGKIWPSYKGRP-GNFLQSRPEPTAPPFLQ SRPEPTAPPEESLRSG-VETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

3. A process according to claim 1, characterized in that a host cell is transformed with an expression vector capable of expressing a polypeptide with the amino acid sequence:
MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-TVATLYCVHQRIEIKDTKEALDKIEEEQNKSK KKAQQAAADTGHSSQVSQNY

4. A process according to claim 1, characterized in that a host cell is transformed with an expression vector capable of expressing a polypeptide with the amino acid sequence:
MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAV-NPGLLETSEGCRQILG QLQPSLQTGSEELRSLYN-TVATLYCVHQRIEIKDTKEALDKIEEEQNKSK KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQA-ISPRTLNAWVKVVEEK AFSPEVIPMFSALSEGATP-QDLNTMLNTVGGHQAAMQMLKETINEEAAEW DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQI-GWMTNNPPIPVGEIY KRWIILGLNKIVRMYSPTSIL-DIRQGPKEPFRDYVDRFYKTLRAEQASQE VKNWMTETLLVQNANPDCKTILKALGPAATLEEM-MTACQGVGGPGHKARV LAEAMSQVTNTATIMM-QRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG CWKCGKEGHQMKDCTERQANFLGKIHRTGVVAM-IA

5. A process according to claim 1, characterized in that a host cell is transformed with an expression vector capable of expressing a polypeptide with the amino acid sequence:
MQRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKK-GCWKCGKEGHQMKDCT ERQANFLGKIWPSYKG-RPGNFLQSRPEPTAPPFLQSRPEPTAPPEESLRS GVETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

6. A process according to claim 1, characterized in that a host cell is transformed with an expression vector capable of expressing a polypeptide with the amino acid sequence:
PIVQNIQGQMVHQAISPRTLNAWVKVVEEKAFSPE-VIPMFSALSEGATPQ DLNTMLNTVGGHQAAMQM-LKETINEEAAEWDRVHPVHAGPIAPGQMREPR GSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIIL-GLNKIVRMYSPTSI LDIRQGPKEPFRDYVDRFYKT-LRAEQASQEVKNWMTETLLVQNANPDCKT ILKAL-GPAATLEEMMTACQGVGGPGHK

7. A process according to any one of claims 1 to 6, characterized in that as a host cell a yeast cell is used.

8. A process according to claim 7, characterized in that as a yeast cell a S. cerevisiae cell is used.

9. A process for producing polypeptides immunologically equivalent to the gag-protein products of HTLV-III comprising: transforming a yeast cell with an expression vector comprising a gene coding for said gag-protein products operably linked to a promoter sequence enabling transcription, translation and expression of said gag-protein products in said yeast cell;

culturing said yeast cell so that the protein products are expressed; and,

extracting and isolating said protein products.

10. A process for the preparation of an expression vector capable in a host cell of effecting the expression of a polypeptide as defined in any one of claims 1 to 6, which process comprises isolating a gene coding for said polypeptides and operably linking said gene with a promoter sequence.

11. A process according to claim 10, characterized in that a promoter sequence capable of effecting expression in a yeast cell is used.

12. A process according to claim 11, characterized in that a promoter sequence capable of effecting expression in S. cerevisiae cell is used.

13. A process according to claim 12, characterized in that a PHO5 promoter sequence is used.

14. A process according to any one of claims 10 to 13, characterized in that as a gene the ClaI to EcoRI restriction size fragment of the λHXB-3 genome is used.

15. A process according to any one of claims 10 to 13, characterized in that as a gene the ClaI to BglII restriction site fragment of the ClaI to EcoRI restriction site fragment of the λHXB-3 genome is used.

16. A process according to any one of claims 10 to 13, characterized in that as a gene the ClaI to EcoRI restriction site fragment of λHXB-3, wherein the ClaI to EcoRI fragment is filled in at the BclI restriction site with the base sequence GATC, is used.

17. A process for the preparation of an expression vector capable in a yeast cell of effecting the expression of polypeptides immunologically equivalent to the gag-protein products of HTLV-III, which process comprises isolating a gene coding for said polypeptides and operably linking said gene with a promoter sequence.

18. A process for the preparation of a transformed cell carrying an expression vector capable in a host cell of effecting the expression of a polypeptide as defined in any one of claims 1 to 6, which process comprises transforming a host cell with said expression vector by methods known in the art.

19. A process according to claim 18, characterized in that as a host cell a yeast cell is used.

20. A process according to claim 19, characterized in that as a yeast cell a S. cerevisiae cell is used.

21. A process according to claim 20, characterized in that as a S. cerevisiae cell S. cerevisiae 20B-12 is used.

22. A process for the preparation of a transformed yeast cell carrying an expression vector capable in a host cell of effecting the expression of polypeptides immunologically equivalent to the gag-protein products of HTLV-III, which process comprises transforming a yeast cell with said expression vector by methods known in the art.

23. A process for testing human blood for the presence of antibodies to the viral etiologic agent of AIDS which method comprises mixing a composition containing a polypeptide as defined in any one of claims I to 6 or 9 or mixtures thereof with a sample of human blood and determining whether said protein or any of its natural proteolytic proteins or mixtures thereof binds to AIDS antibodies present in the blood sample.

24. A process for the preparation of a vaccine comprising mixing a polypeptide as defined in any one of claims I to 6 or 9 with a physiologically acceptable carrier.

25. A process for the preparation of antibodies against AIDS virus comprising injecting a mammalin or avian animal with a sufficient amount of a polypeptide as defined in any one of claims I to 6 or 9 and recovering said antibodies from the serum of said animals.

26. Antibodies raised against a polypeptid as defined in any one of claims I to 6 or 9.

27. The antibodies of claim 26 which are monoclonal antibodies.

28. A polypeptide immunoloqically equivalent to the gag-protein products of HTLV-III whenever prepared by a process as claimed in any one of claims I to 9.

29. A gene containing a gene portion having a DNA sequence encoding a polypeptide as defined in any one of claims I to 6 operably linked to a promoter capable of effecting the expression of said DNA sequence.

30. The gene of claim 29 wherein said gene portion is the ClaI to EcoRI restriction site fragment of the λ HXB-3 genome.

3l. The gene of claim 30 wherein the promoter is the Bam HI to Aha III restriction site fragment of PHO5.

32. The gene of claim 29 wherein said gene portion is the ClaI to BglII restriction site fragment of the ClaI to EcoRI fragment of the λ HXB-3 genome.

33. The gene of claim 32 wherein the promoter is the BamHI to Aha III restriction site fragment of PHO5.

34. The gene of claim 29 where the gene portion is the ClaI to EcoRI restriction site fragment of λ HXB-3, wherein the ClaI to EcoRI fragment is filled in at its BclI restriction site with the base sequence GATC.

35. The gene of claim 34 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

36. A gene containing a gene portion having a DNA sequence encoding the gag-protein products of HTLV-III operably linked to a promoter capable of effecting the expression of said DNA sequence in yeast.

37. A recombinant expression vector capable of effecting the expression of a polypeptide as defined in any one of claims I to 6 containing a gene portion having a DNA sequence encoding said polypeptide operably linked to a promoter capable of effecting the expression of said DNA sequence.

38. A vector according to claim 37 wherein the gene portion is the ClaI to EcoRI restriction fragment of the λ HXB-3 genome.

39. A vector according to claim 38 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

40. A vector according to claim 37 wherein the gene portion is the ClaI to BglII restriction site fragment of the Cla I to EcoRI restriction site fragment of the λ HXB-3 genome.

4l. A vector according to claim 40 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

42. A vector according to claim 37 wherein the gene portion is the ClaI to EcoRI restriction site fragment of λ HXB-3, wherein the ClaI to EcoRI fragment is filled in at the BclI restriction site with the base sequence GATC.

43. A vector according to claim 42 wherein the promoter is the BamHI to AhaIII restriction site fragment of PHO5.

44. A vector according to claim 39 which is pYE72/gagl.

45. A vector according to claim 4l which is pYE72/gag2.

46. A vector according to claim 43 which is pYE72/gag3.

47. A recombinant expression vector capable of effecting the expression of the gag-protein products of HTLV-III in yeast containing a gene portion having a DNA sequence encoding the gag-protein of HTLV-III operably linked to a promoter capable of effecting the expression of said DNA sequence.

48. A transformed cell carrying a vector as claimed in any one of claims 37 to 46.

49. A transformed cell according to claim 48 which is a yeast cell.

50. A transformed cell according to claim 49 which is a S. cereviviae yeast cell.

5l. A transformed cell according to claim 50 which is S. cerevisiae 20B-l2.

52. The use of a polypeptide as defined in any one of claims I to 6 or 9 for the preparation of a protective immunization vaccine.

53. The use of a polypeptide as defined in any one of claims I to 6 or 9 for the preparation of antibodies against AIDS virus.

54. The use of a polypeptide as defined in any one of claims I to 6 or 9 for testing human blood for the presence of AIDS virus.

55. A test kit for the determination of antibodies against AIDS virus comprising in a container a polypeptide as defined in any one of claims I to 6 or 9.

56. A test kit for the determination of AIDS virus comprising in a container antibodies against AIDS virus elicited by a polypeptide as defined in any one of claims I to 6 or 9.

Figure 1-A

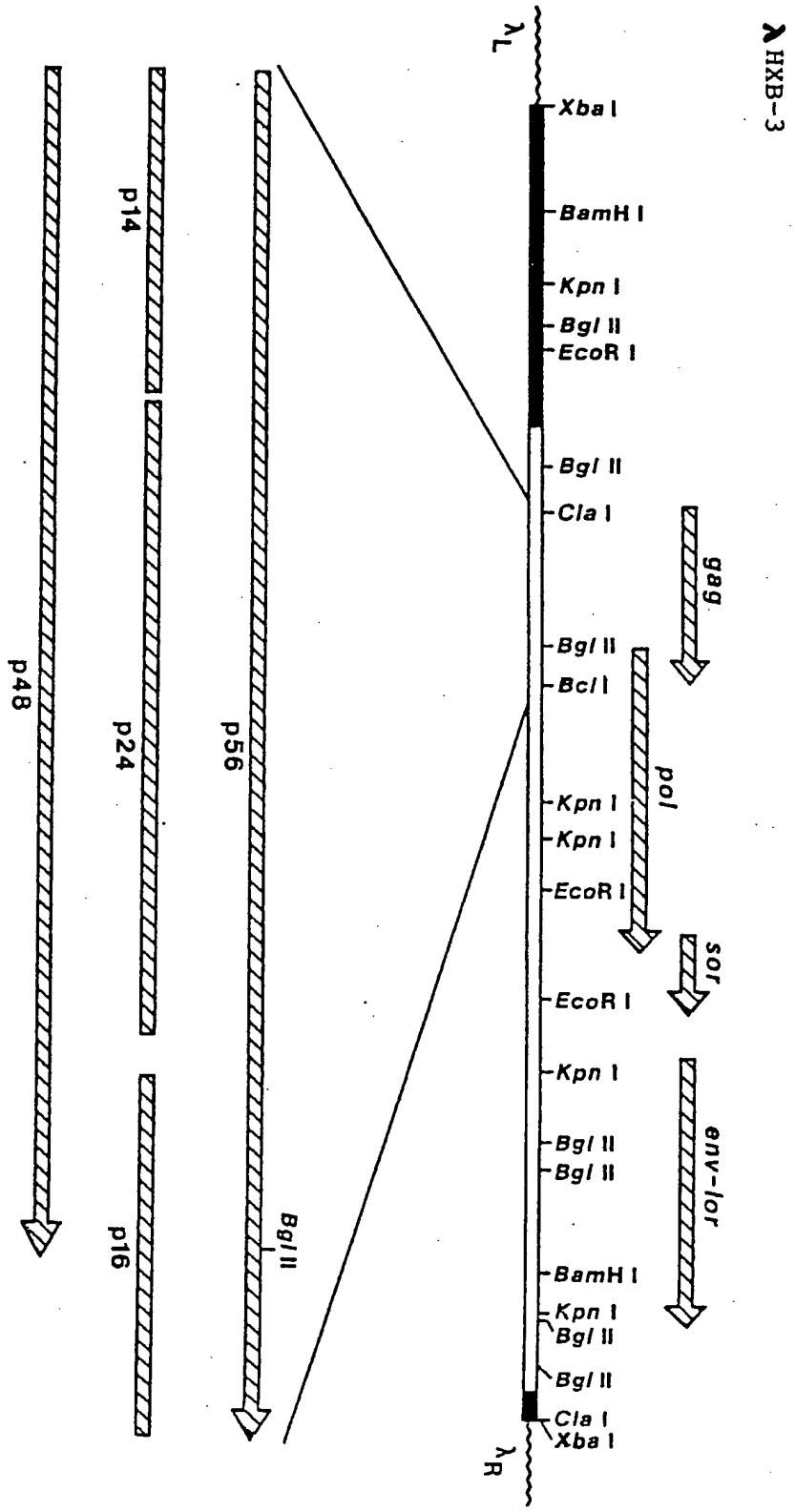

λL
HXB-3
Xba I
BamH I
Kpn I
Bgl II
EcoR I
Bgl II
Cla I
gag
Bgl II
Bcl I
pol
Kpn I
Kpn I
EcoR I
sor
EcoR I
Kpn I
env-lor
Bgl II
Bgl II
BamH I
Kpn I
Bgl II
Bgl II
Cla I
Xba I
λR
p14
p48
p24
p56
p16
Bgl II

Figure 1-B

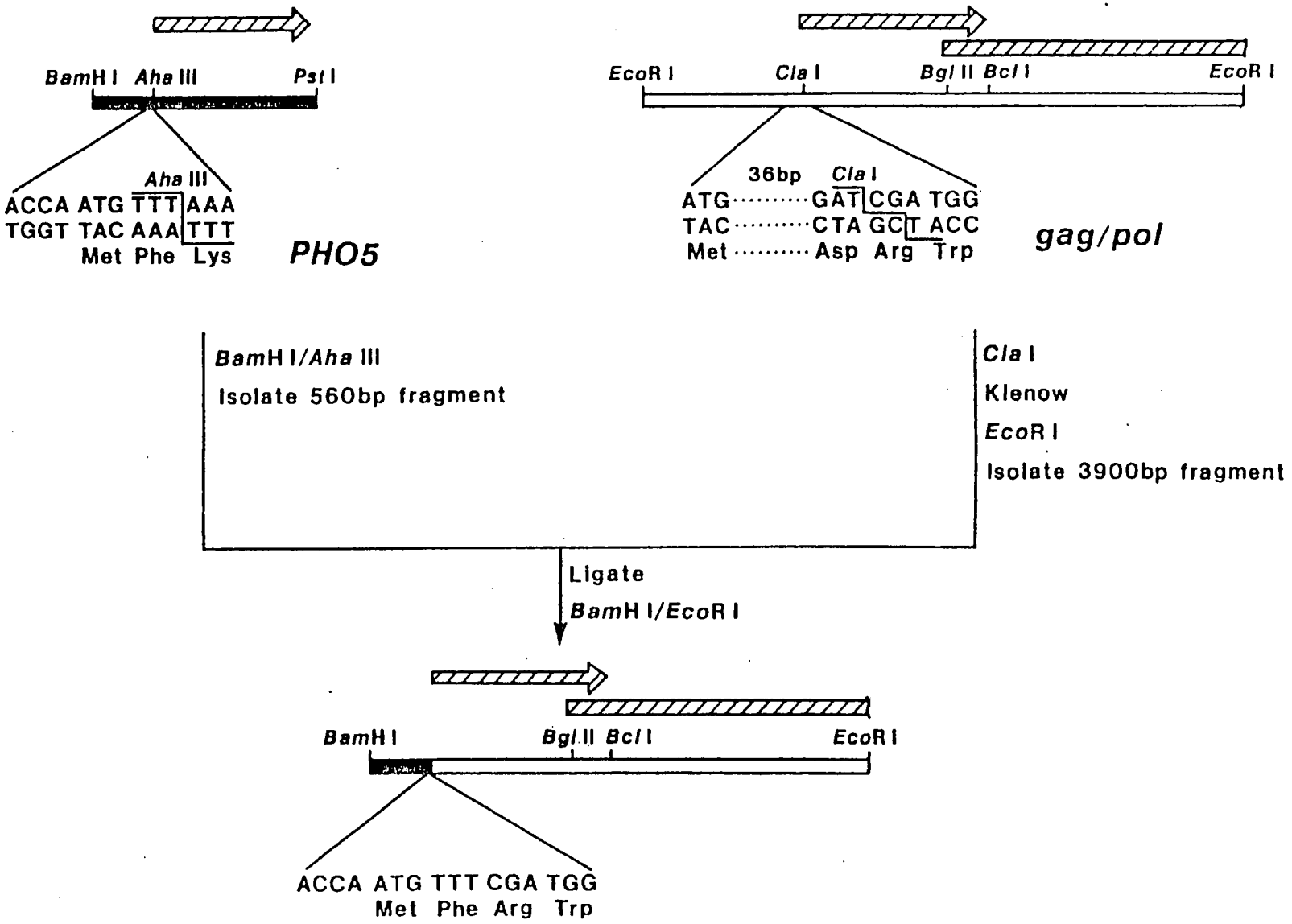

BamH I
Aha III
Pst I
Aha III
ACCA ATG TTT AAA
TGGT TAC AAA TTT
Met Phe Lys
PHO5
EcoR I
Cla I
Bgl II Bcl I
EcoR I
36bp
Cla I
ATG·····GAT CGA TGG
TAC·····CTA GCT ACC
Met·····Asp Arg Trp
gag/pol
BamH I/Aha III
Isolate 560bp fragment
Cla I
Klenow
EcoR I
Isolate 3900bp fragment
Ligate
BamH I/EcoR I
BamH I
Bgl II Bcl I
EcoR I
ACCA ATG TTT CGA TGG
Met Phe Arg Trp

Figure 2

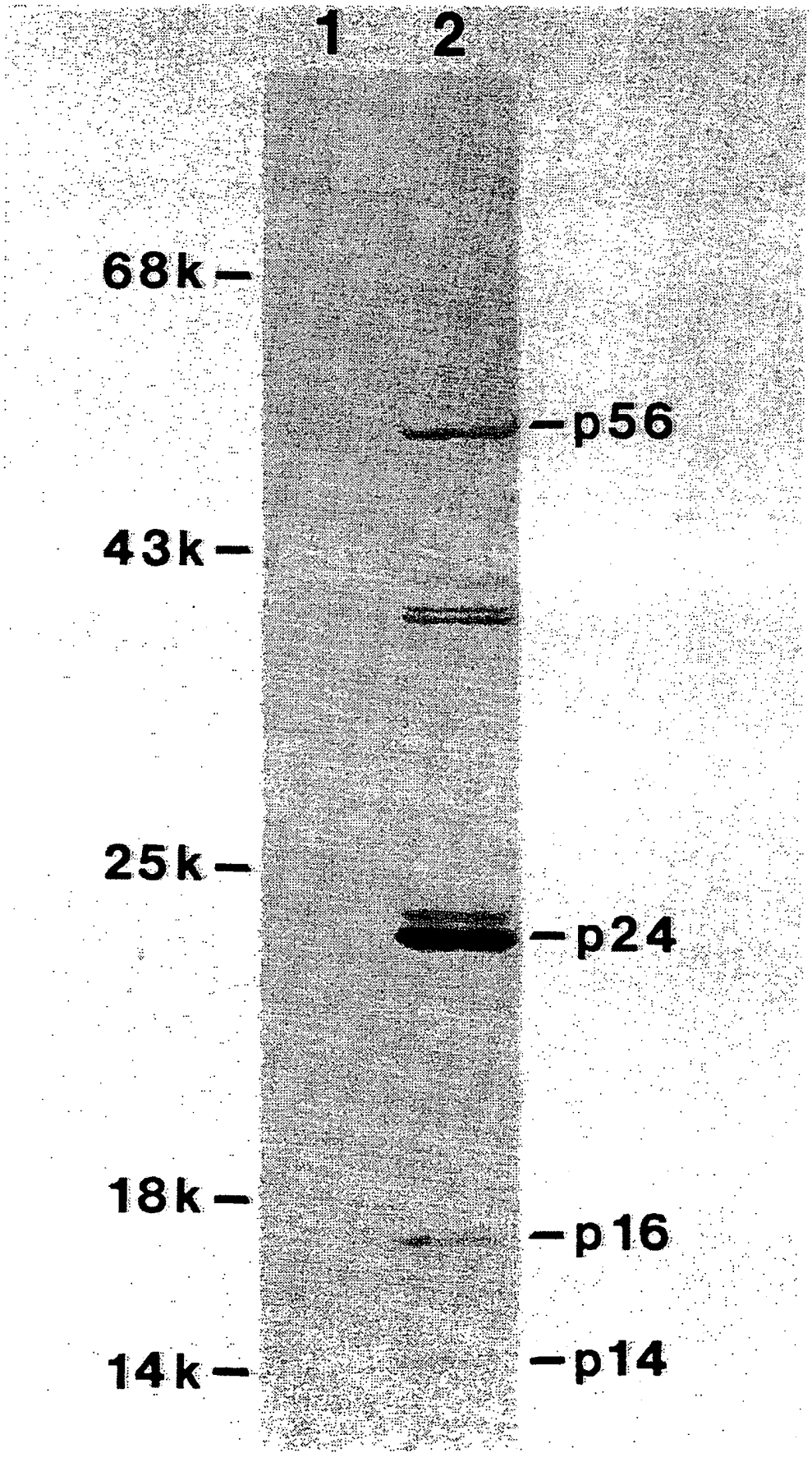


Figure 3

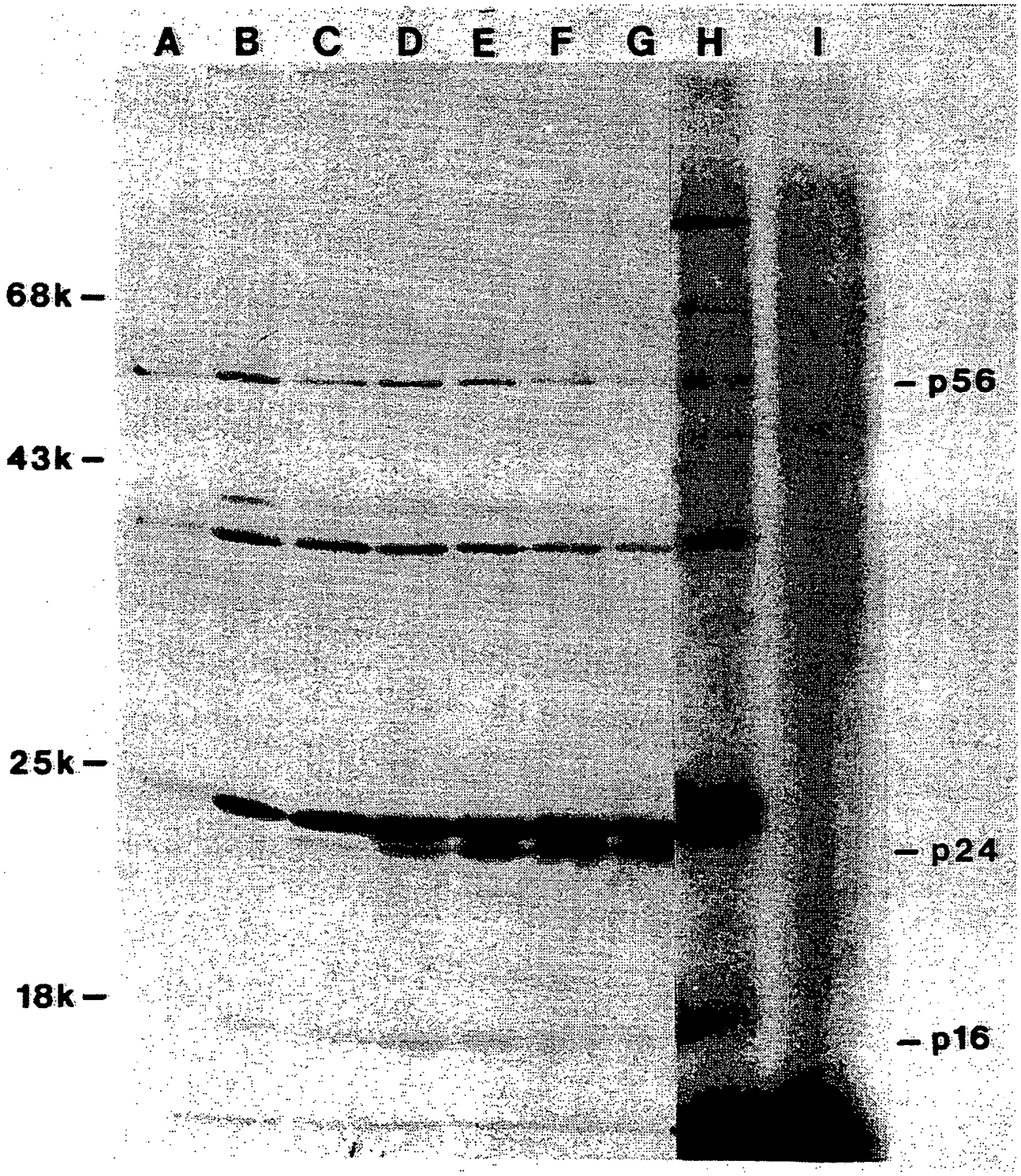


FIGURE 4

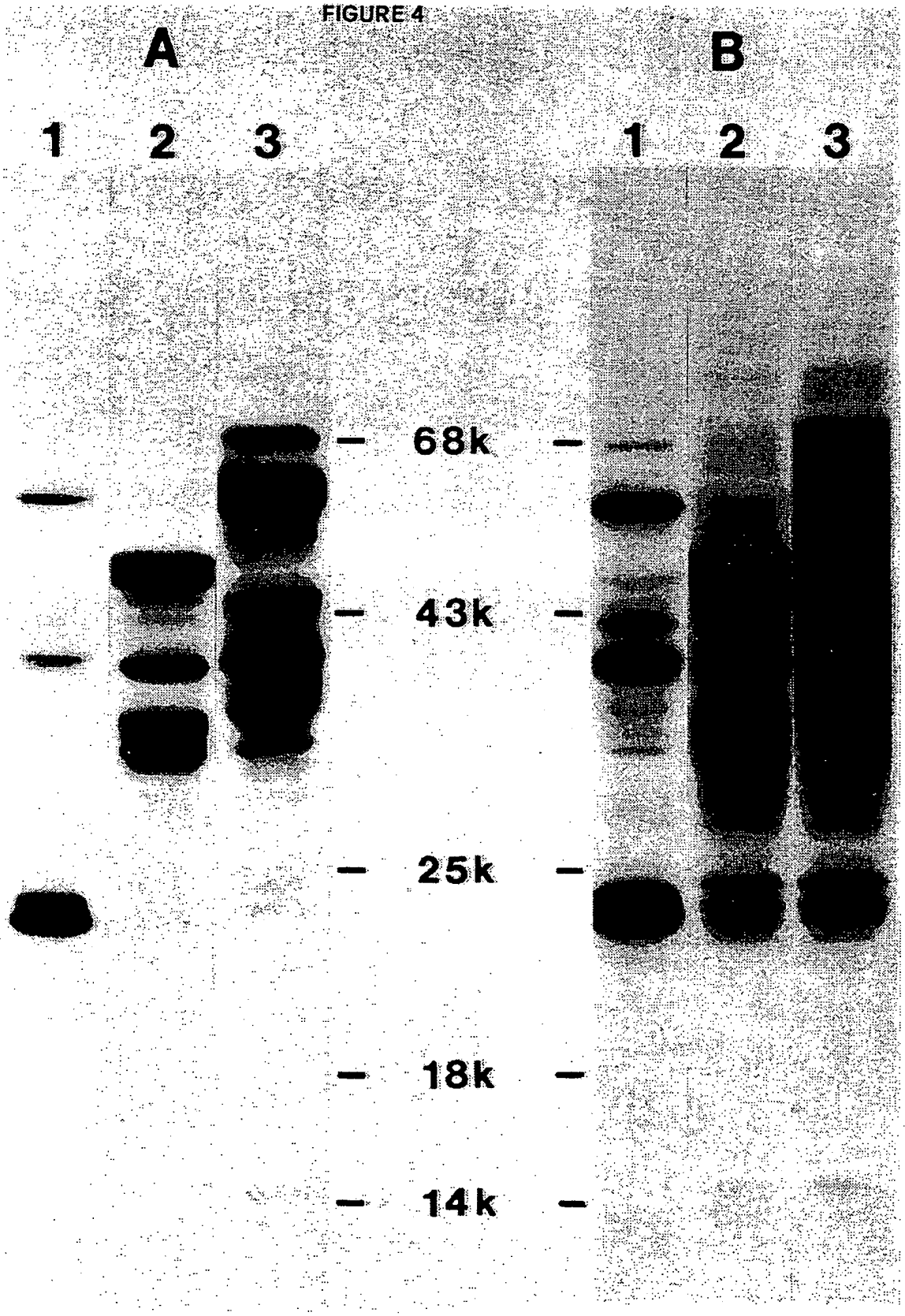
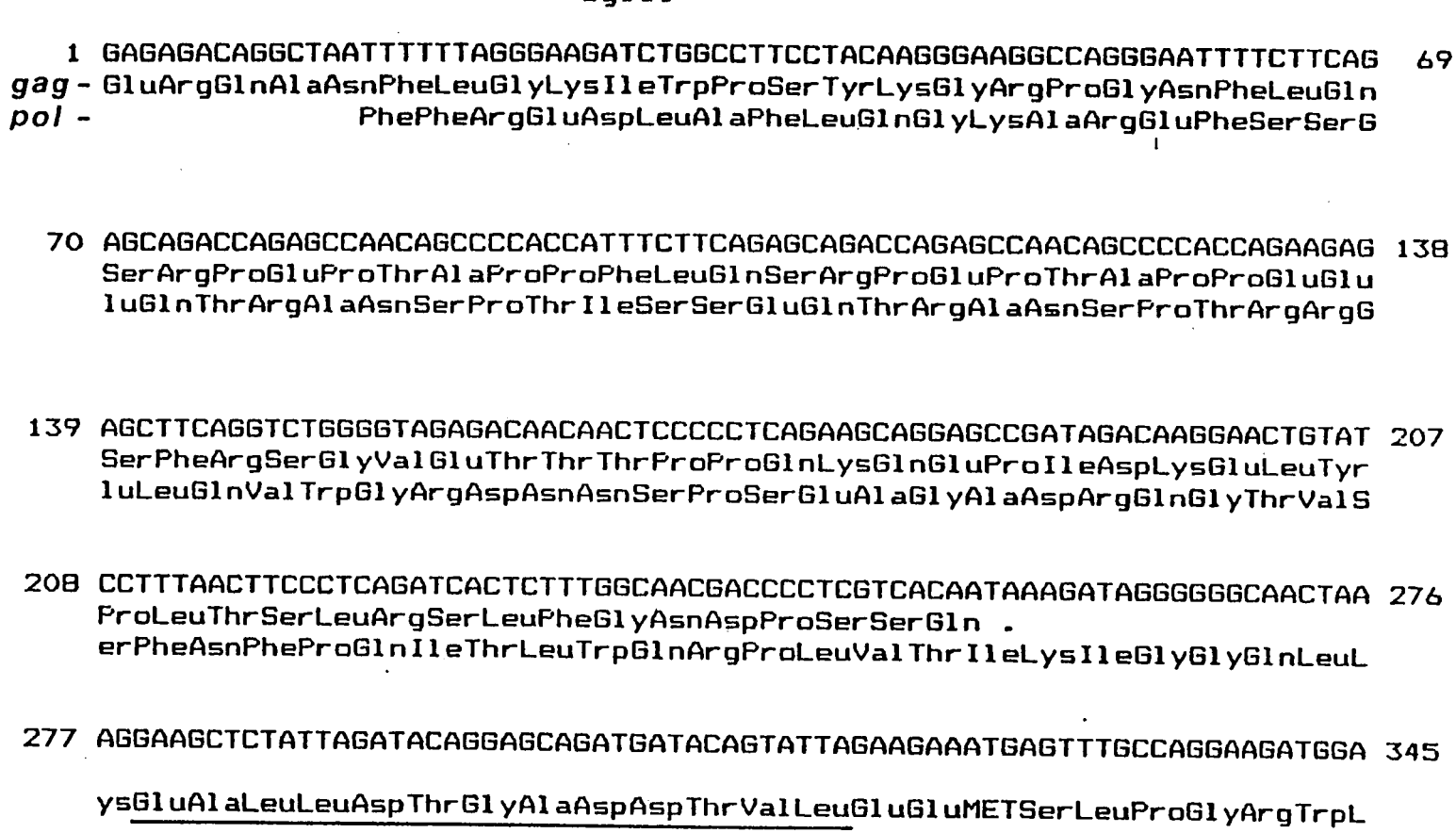


Figure 5


BglII
1 GAGAGACAGGCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCAG 69
gag - GluArgGlnAlaAsnPheLeuGlyLysIleTrpProSerTyrLysGlyArgProGlyAsnPheLeuGln
pol - PhePheArgGluAspLeuAlaPheLeuGlnGlyLysAlaArgGluPheSerSerG
70 AGCAGACCAGAGCCAACAGCCCCACCATTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCAGAAGAG 138
SerArgProGluProThrAlaProProPheLeuGlnSerArgProGluProThrAlaProProGluGlu
luGlnThrArgAlaAsnSerProThrIleSerSerGluGlnThrArgAlaAsnSerProThrArgArgG
139 AGCTTCAGGTCTGGGGTAGAGACAACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTAT 207
SerPheArgSerGlyValGluThrThrThrProProGlnLysGlnGluProIleAspLysGluLeuTyr
luLeuGlnValTrpGlyArgAspAsnAsnSerProSerGluAlaGlyAlaAspArgGlnGlyThrValS
208 CCTTTAACTTCCCTCAGATCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAA 276
ProLeuThrSerLeuArgSerLeuPheGlyAsnAspProSerSerGln .
erPheAsnPheProGlnIleThrLeuTrpGlnArgProLeuValThrIleLysIleGlyGlyGlnLeuL
277 AGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGA 345
ysGluAlaLeuLeuAspThrGlyAlaAspAspThrValLeuGluGluMETSerLeuProGlyArgTrpL

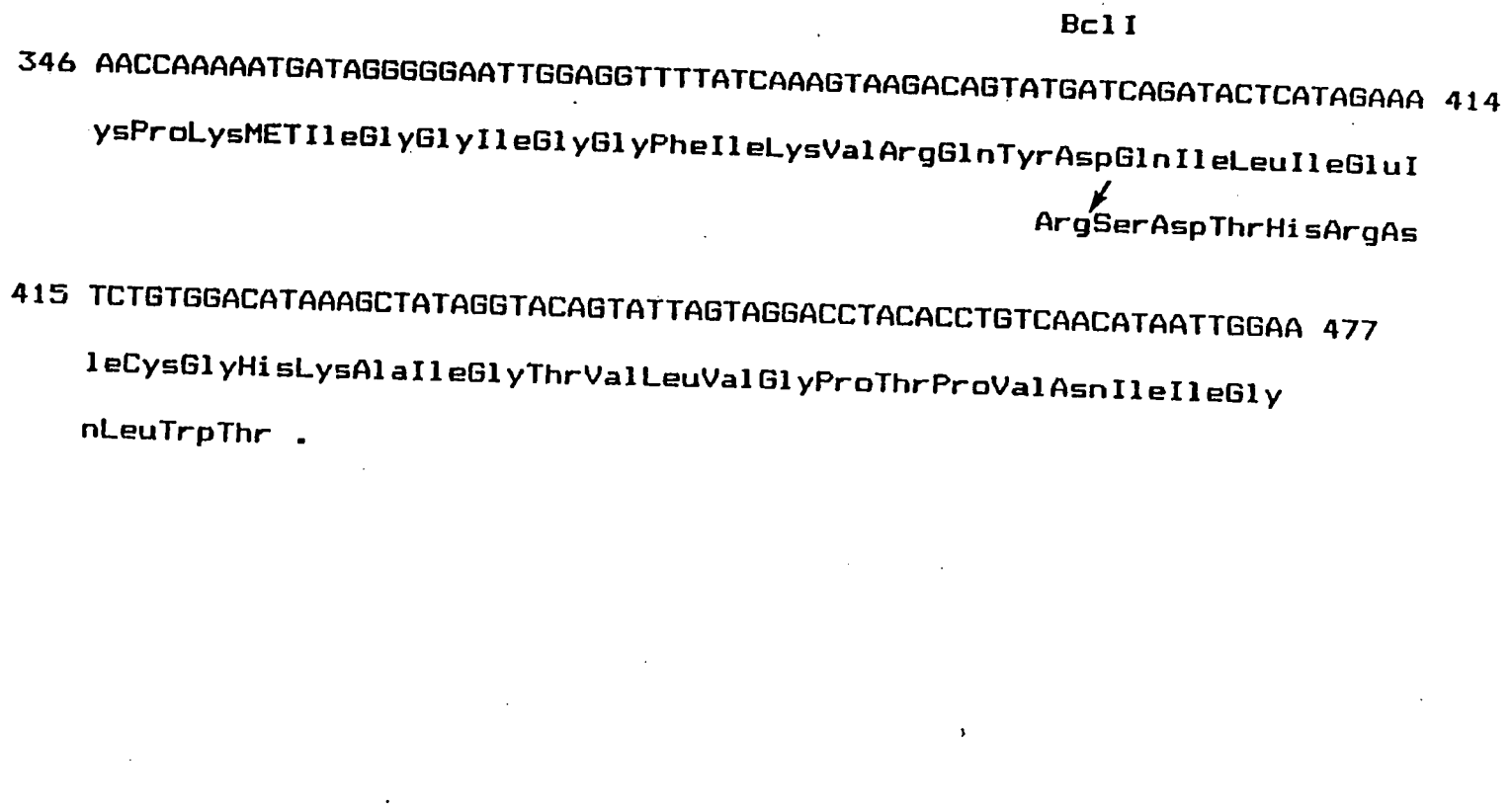


Figure 5 (cont.)

Figure 6-A

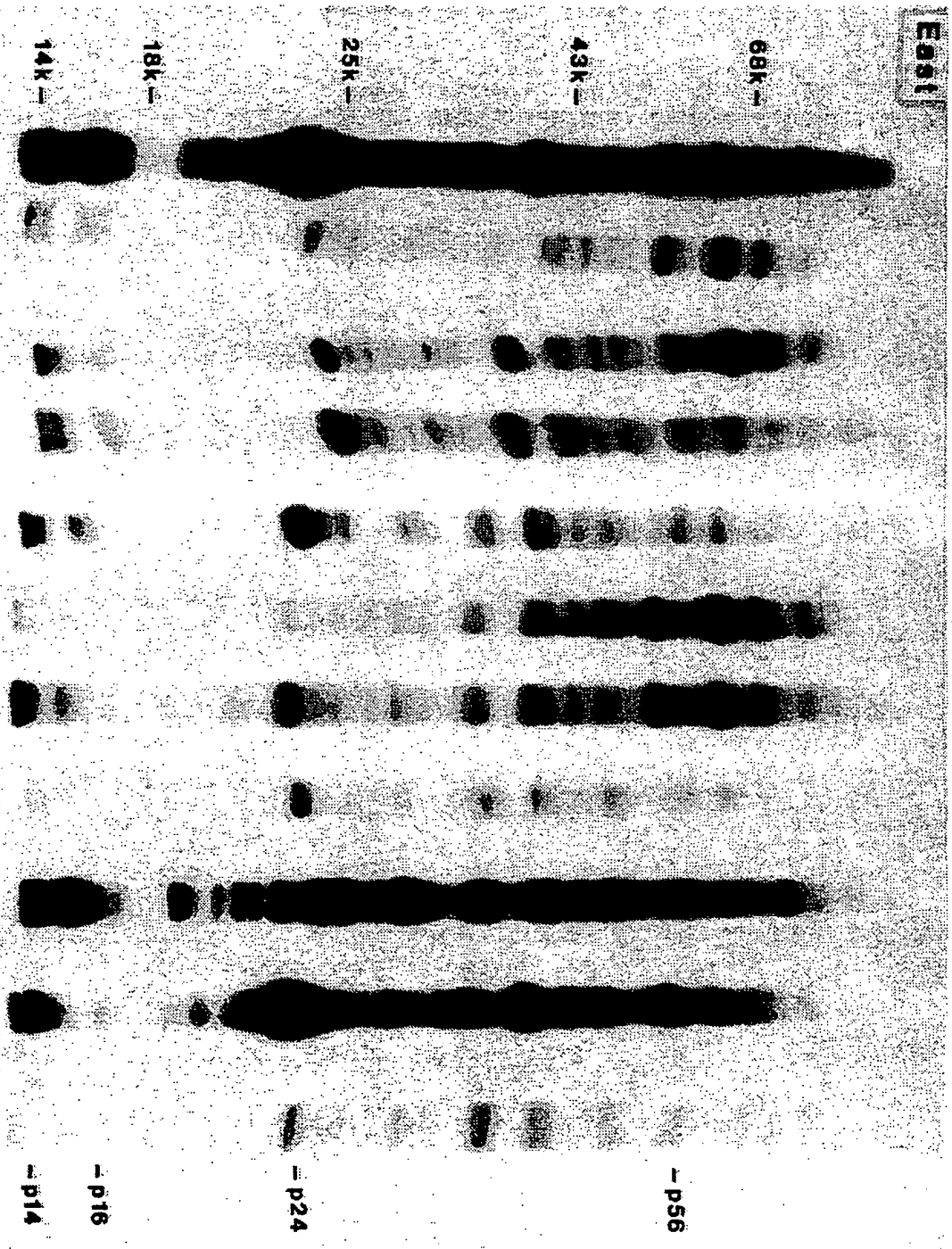


Figure 6-B

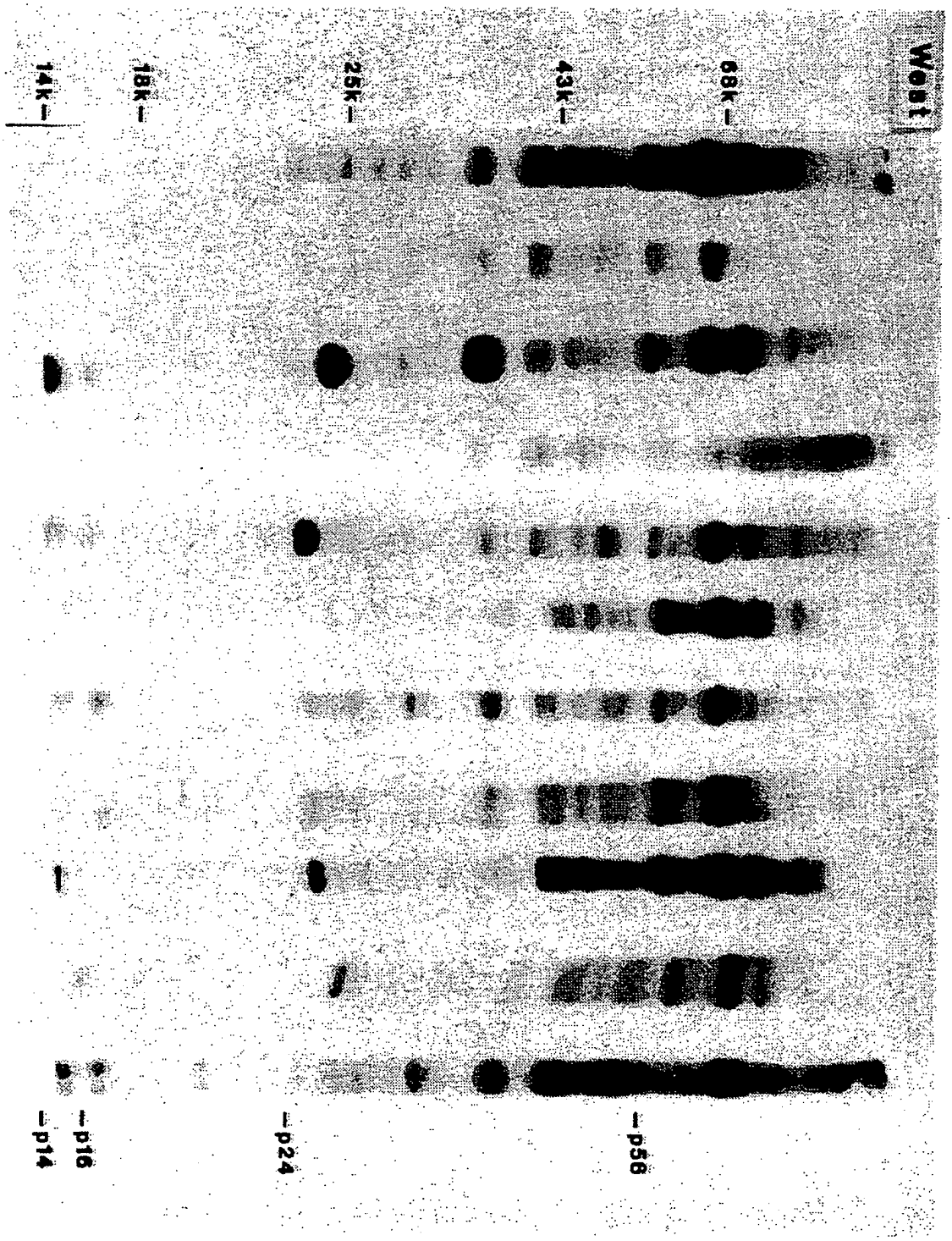


FIGURE 7

GAG 56 SEQ

ATGTTTCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACA
TATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACAT
CAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAA
CTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAA
AGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
AGCAAGCAGCAGCTGACACAGGACACAGCAGTCAGGTCAGCCAAAATTACCCTATAGTGCAG
AACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAA
AGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTAATACCCATGTTTTCAGCATTATCAGAAG
GAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATG
CAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTACATCCAGTGCA
TGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTA
CTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAA
ATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAG
CATTCTGGACATAAGACAAGGACCAAAAGAACCCTTTAGAGACTATGTAGACCGGTTCTATA
AAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAATTGGATGACAGAAACCTTGTTG
GTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCAGCTACACT
AGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGG
CTGAAGCAATGAGCCAAGTAACAAATACAGCTACCATAATGATGCAGAGAGGCAATTTTAGG
AACCAAAGAAAGATTGTTAAGTGTTTCAATTGTGGCAAAGAAGGGCACATAGCCAGAAATTG
CAGGGCCCCTAGGAAAAAGGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAAGATT
GTACTGAGAGACAGGCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGG
AATTTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCATTTCTTCAGAGCAGACCAGAGCC
AACAGCCCCACCAGAAGAGAGCCTCAGGTCTGGGGTAGAGACAACAACTCCCTCTCAGAAGC
AGGAGCCGATAGACAAGGAACTGTATCCTTTAACTTCCCTCAGATCACTCTTTGGCAACGAC
CCCTCGTCACAATAA

FIGURE 8

GAG56.PEP

P56

MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQILG
QLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQAISPRTLNAWVKVVEEK
AFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQAAMQMLKETINEEAAEW
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTNNPPIPVGEIY
KRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQE
VKNWMTETLLVQNANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARV
LAEAMSQVTNTATIMMQRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG
CWKCGKEGHQMKDCTERQANFLGKIWPSYKGRPGNFLQSRPEPTAPPFLQ
SRPEPTAPPEESLRSGVETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

FIGURE 9

GAG24.SEQ

CCTATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAA
TGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTAATACCCATGTTTTCAG
CATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACAT
CAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGT
ACATCCAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACA
TAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATC
CCAGTAGGAGAAATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTA
TAGCCCTACCAGCATTCTGGACATAAGACAAGGACCAAAAGAACCCTTTAGAGACTATGTAG
ACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAATTGGATGACA
GAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACC
AGCAGCTACACTAGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAG

FIGURE 10

GAG24.PEP
P24
PIVQNIQGQMVHQAISPRTLNAWVKVVEEKAFSPEVIPMFSALSEGATPQ
DLNTMLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMREPR
GSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSI
LDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKNWMTETLLVQNANPDCKT
ILKALGPAATLEEMMTACQGVGGPGHK

FIGURE 11

GAG16.SEQ

ATGCAGAGAGGCAATTTTAGGAACCAAAGAAAGATTGTTAAGTGTTTCAATTGTGGCAAAGA
AGGGCACATAGCCAGAAATTGCAGGGCCCCTAGGAAAAAGGGCTGTTGGAAATGTGGAAAGG
AAGGACACCAAATGAAAGATTGTACTGAGAGACAGGCTAATTTTTTTAGGGAAGATCTGGCCT
TCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCATT
TCTTCAGAGCAGACCAGAGCCAACAGCCCCACCAGAAGAGAGCCTCAGGTCTGGGGTAGAGA
CAACAACTCCCTCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTATCCTTTAACTTCCCTC
AGATCACTCTTTGGCAACGACCCCTCGTCACAA

FIGURE 12

GAG16.PEP
P16
MQRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKGCWKCGKEGHQMKDCT
ERQANFLGKIWPSYKGRPGNFLQSRPEPTAPPFLQSRPEPTAPPEESLRS
GVETTTPSQKQEPIDKELYPLTSLRSLFGNDPSSQ

FIGURE 13

GAG14.SEQ

ATGTTTCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACA
TATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACAT
CAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAA
CTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAA
AGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
AGCAAGCAGCAGCTGACACAGGACACAGCAGTCAGGTCAGCCAAAATTAC

FIGURE 14

GAG14.PEP
P14
MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQILG
QLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNY

FIGURE 15

GAG48.SEQ

ATGTTTCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACA
TATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACAT
CAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAA
CTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAA
AGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
AGCAAGCAGCAGCTGACACAGGACACAGCAGTCAGGTCAGCCAAAATTACCCTATAGTGCAG
AACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAA
AGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTAATACCCATGTTTTCAGCATTATCAGAAG
GAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATG
CAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTACATCCAGTGCA
TGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTA
CTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAA
ATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAG
CATTCTGGACATAAGACAAGGACCAAAAGAACCCTTTAGAGACTATGTAGACCGGTTCTATA
AAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAATTGGATGACAGAAACCTTGTTG
GTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCAGCTACACT
AGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGG
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AACCAAAGAAAGATTGTTAAGTGTTTCAATTGTGGCAAAGAAGGGCACATAGCCAGAAATTG
CAGGGCCCCTAGGAAAAAGGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAAGATT
GTACTGAGAGACAGGCTAATTTTTTTAGGGAAGATCCACAGGACGGGTGTGGTCGCCATGATC
GCGTAG

FIGURE 16

GAG48.PEP

P48

MFRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQILG
QLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNKSK
KKAQQAAADTGHSSQVSQNYPIVQNIQGQMVHQAISPRTLNAWVKVVEEK
AFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQAAMQMLKETINEEAAEW
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTNNPPIPVGEIY
KRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQE
VKNWMTETLLVQNANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARV
LAEAMSQVTNTATIMMQRGNFRNQRKIVKCFNCGKEGHIARNCRAPRKKG
CWKCGKEGHQMKDCTERQANFLGKIHRTGVVAMIA

European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 87 10 0064

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 82, no. 22, November 1985, pages 7748-7752, Washington, US; D.J. DOWBENKO et al.: "Bacterial expression of the acquired immunodeficiency syndrome retrovirus p24 gag protein and its use as a diagnostic reagent" * Pages 7749-7751 *	1,6,8, 16,34, 38-40	C 12 N 15/00 C 07 K 13/00 C 12 N 1/18 A 61 K 39/21 C 12 P 21/02 G 01 N 33/569 C 07 K 15/00
X	SCIENCE, vol. 228, 31st May 1985, pages 1094-1096; F. BARIN et al.: "Virus envelope protein of HTLV-III represents major target antigen for antibodies in AIDS patients" * Figure 1 *	1,2,5, 6,8,38 -40	
X	SCIENCE, vol. 228, 3rd May 1985, pages 593-595; W.G. ROBEY et al.: "Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients" * Figure 1 *	1,2,6, 8,38- 40	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N A 61 K G 01 N

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	06-04-1987	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 87 10 0064

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	NATURE, vol. 313, 7th February 1985, pages 450-458; M.A. MUESING et al.: "Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus" * Page 452, last paragraph; page 453 *	1,2,6, 8,38-40	
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 82, August 1985, pages 5199-5202; F. DI MARZO VERONESE et al.: "Monoclonal antibodies specific for p24, the major core protein of human T-cell leukemia virus type III" * Page 5201 *	6,8,40 -44	
X,P	EP-A-0 181 150 (CHIRON CORP.) * Pages 30,31 *	1,5,6, 8	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X,P	SCIENCE, vol. 231, 28th March 1986, pages 1580-1584; R.A. KRAMER et al.: "HTLV-III gag protein is precessed in yeast cells by the virus pol-protease" * Whole article *	1-46	
X,P	EP-A-0 187 041 (GENENTECH INC.) * Claims 1-3,10-12,15,16 *	1,8	

The present search report has been drawn up for all claims

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(54) Recombinant immunogenic actinomycetale.

(57) A mycobacteria transformed with an antigen-encoding gene, such as nef, under the control of a Streptomyces stress-responsive promoter, such as the S. albus groES/groEL1 promoter, and preferably associated with a synthetic ribosome binding site. The recombinant mycobacteria can be used as a vaccine against, for example, a pathogen which carries the antigen.

EP 0 521 220 A1

This invention relates to a strain of a species of Actinomycetale (the "Actinomycetale strain"), particularly a strain of mycobacteria, which: a) has been transformed with at least one gene or a DNA fragment thereof (an "antigen-encoding gene") that codes for all or at least part of an antigen, particularly at least one epitope thereof (a "desired antigen", preferably a "immunizing antigen"); and b) can be used to inoculate mammals, particularly humans, to immunize them, preferably protect them (e.g., from a pathogen carrying all or part of the desired antigen). In this regard, the term "desired antigen" comprises any molecule which is capable of inducing an immune response, including a protein, glycoprotein, glycolipoprotein, peptidoglycolipid, etc., or an immunogenic fragment thereof, and which can, for example, come from, or be derived from, a pathogen against which the transformed Actinomycetale strain is to provide an immunity, preferably a protection.

Background of the Invention

Various types of vaccines have been developed against pathogens. When a humoral immune response is able to confer protection, subunit or killed vaccines are efficient. However, in the case of tuberculosis and certain other infectious diseases, killed pathogens are not protective.

The vaccine currently used to protect against tuberculosis, Mycobacterium bovis BCG (Bacille Calmette-Guerin) or "BCG", is the unique live bacterial vaccine in use. M. bovis BCG and all other mycobacteria survive in macrophages which are antigen-presenting cells and initiate the humoral and T-cell mediated immune response (EDWARDS and KIRKPATRICK, 1986). This might explain the stimulant activities of this vaccine. M. bovis BCG offers many advantages for development of a recombinant polyvalent vaccine vector expressing antigens from a wide variety of pathogens, particularly those in which cell-mediated immunity is important for protection (BLOOM, 1986; JACOBS et al, 1988). It is an attenuated M. bovis strain which has been used without major side effects to vaccinate more than two billion people, it is produced at low cost, and it can be given at birth as a single dose and is then able to confer long term immunity. It also has stimulant activities and has been used as an adjuvant in various protocols of immunization.

The recent development of genetic tools for transforming mycobacteria has enabled the cloning of foreign genes in both fast-growing (M. smegmatis) and slow-growing (M. bovis BCG) strains. Several phasmid- and plasmid-based vectors have been reported, for example in PCT publication W088/06626. Starting from pAL5000, a plasmid from M. fortuitum whose entire nucleotide sequence has been determined (RAUZIER et al, 1988), various E. coli-mycobacteria shuttle plasmids have been constructed which stably replicate in mycobacteria, including a "mini" mycobacterial replicon, pRR3 (RANES et al, 1990). Foreign genes have been cloned on these vectors and on other integrative vectors described in STOVER et al (1991) and shown to be expressed in mycobacteria using their own control elements or as fused genes (MATSUO et al, 1990).

Summary of the Invention

This invention provides an immunogenic Actinomycetale strain, particularly a strain of mycobacteria, transformed with the following, operably linked, chimaeric DNA sequence, comprising an antigen-encoding gene that: a) is foreign to the Actinomycetale strain; b) encodes a desired antigen, preferably an immunizing antigen; c) is under the control of a promoter foreign to the Actinomycetale strain, preferably a promoter from another strain of Actinomycetale, especially a promoter from another species of Actinomycetale, particularly a Streptomyces promoter, quite particularly a stress-responsive (e.g., heat-shock) promoter; and d) is preferably associated with a ribosome binding site ("RBS") foreign to the Actinomycetale strain, particularly foreign to Actinomycetale and/or synthetic.

This invention also provides the aforementioned chimaeric DNA sequence, especially wherein the antigen-encoding gene codes for an HIV-1 protein, particularly the Nef protein, or an antigenic fragment or an epitope thereof which can induce an immune response in a mammal, particularly a T or B response, quite particularly wherein the antigen-encoding gene is under the control of the S. albus groES/groEL1 promoter.

This invention further provides a system, such as a plasmid, capable of transforming a Actinomycetale strain. This system comprises a foreign promoter, a foreign ribosome binding site and a DNA fragment coding for an antigen foreign to the Actinomycetale strain, particularly the aforementioned chimaeric DNA sequence, quite particularly pWRIP17.

This invention yet further provides: a) an immunogenic composition, particularly a vaccine, comprising the aforementioned transformed Actinomycetale strain, particularly a strain of mycobacteria, quite particularly BCG, and if necessary, a suitable carrier, as well as, for example, an adjuvant for human use; and b) a method of immunizing a mammalian host, particularly a human, against, for example, a pathogen, comprising the step of administering to the host the aforemen-

tioned composition.

This invention also provides: a) a process to produce the desired antigen by culturing the transformed Actinomycetale strain; and b) a process for using the desired antigen or the transformed Actinomycetale strain, for example, as a diagnostic agent and/or an immunogenic agent. An antigenic preparation containing the desired antigen or the transformed Actinomycetale strain can induce the synthesis of specific antibodies or cellular reactions in in vitro and in vivo diagnostic tests and immunogenic treatments.

Detailed Description of the Invention

In accordance with this invention, a recombinant immunogenic strain of a species of Actinomycetale, such as Actinomycetaceae and Mycobacteriacae, especially Corynebacteria, Streptomyces and Mycobacteria, particularly Mycobacteria, can be obtained by transforming the Actinomycetale strain with the operably linked chimaeric DNA sequence of this invention.

The antigen-encoding gene of the chimaeric DNA sequence or expression cassette, used to transform the Actinomycetale strain in accordance with this invention, can be any gene or DNA fragment thereof which encodes a desired antigen as hereinbefore defined, whose expression products can be used, for example, to induce an immune response, preferably in a vaccine administered to a mammalian host, preferably a human, to immunize and preferably protect the host, for example, against a pathogen. Pathogens, whose immunizing antigens can be encoded by the antigen-encoding gene, include, for example, viral, parasitic and bacterial, particularly viral, pathogens such as M. leprae, M. tuberculosis, M. intracellulare, M. africanum, M. avium, plasmodium sporozoites and merozoites, diphtheria toxoid, tetanus toxoids, Leishmania, Salmonella, some Treponema, pertussis toxin and other antigenic determinants and viruses, including measles, mumps, herpes, influenza, Schistosoma, Shigella, Neisseria, Borrelia, rabies, polio virus, hepatitis virus, human immunodeficiency virus (HIV), HTLV-I, HTLV-II, and Simian immunodeficiency virus (SIV), as well as oncogenic viruses. Alternatively, the antigen-encoding gene can encode an immunizing antigen from other than a pathogen, such as a snake or insect venom. In this regard, this invention is not limited to the expression of desired antigens for immunizing a mammal against a pathogen but also includes the use of the transformed Actinomycetale strain of this invention for the development of other kinds of immunotherapy treatments, as well as for the production of molecules of interest. For example, the transformed Actinomycetale strain, cloned with a gene coding for the synthesis of a hormone, could be used in an anti-fertility vaccine, or the transformed Actinomycetale strain, cloned with a tumor-associated gene such as an oncogene, could be used in an anti-cancer vaccine.

The promoter, which is upstream (i.e., 5') of, and used to control, the antigen-encoding gene in the chimaeric DNA sequence of this invention is critical to this invention in providing high levels of expression of the gene in Actinomycetale, particularly mycobacteria. The promoter can be any promoter from a strain different from that of the Actinomycetale strain being transformed but is preferably from a different strain of Actinomycetale, particularly from a different species of Actinomycetale. Preferably for the transformation of mycobacteria, the promoter is a Streptomyces promoter. The promoter is also preferably a stress-responsive promoter, especially a heat-shock promoter, particularly an Actinomycetale promoter, quite particularly a groEL promoter or a groEL-like promoter of French patent application 9011186, filed September 10, 1990 (which is incorporated herein by reference). A copy of the Disclosure and Figures of French application 9011186 is appended hereto as "Appendix A", following the list of references.

However, other promoters, not from Actinomycetale, can also be used in the chimaeric DNA sequence of this invention. For example, a phage lambda (pL) promoter (GUY et al, 1987) or a promoter of an antibiotic resistance gene, such as the bacterial promoter of the kanamycin resistance gene (OKA et al, 1981), the bacterial promoter of the sulfonamide gene (MARTIN et al, 1990) or the bacterial TAC promoter (AMRANN et al, 1983), as well as the promoters of other bacterial genes, can be used.

The chimaeric DNA sequence of this invention optionally includes, downstream (i.e., 3') of the promoter and upstream of the antigen-encoding gene, a ribosome binding site (RBS) which is preferably synthetic or foreign to the Actinomycetale strain, particularly the Actinomycetale species, being transformed. It is particularly preferred that the ribosome binding site be foreign to Actinomycetale, generally, and quite particularly preferred that the ribosome binding site for transforming mycobacteria be the synthetic ribosome binding site of the E. coli plasmid pTG1166 (GUY et al, 1987). This particularly preferred RBS has the following sequence:

5'ATCGATAACAGAGGAACAGATCT3'.

Preferably associated with the chimaeric DNA sequence of this invention in the recombinant Actinomycetale strain is a selectable marker gene, such as an antibiotic resistance-encoding gene, for example a gene encoding kanamycin resistance, viomycin resistance, thiostrepton resistance, hyg-

romycin resistance or bleomycin resistance, in order to make it possible to identify and isolate recombinant Actinomycetale strains of this invention. A conventional selectable marker can be used such as is described in PCT publication WO 88/06626 and in European patent publication ("EP") 400,973. The selectable marker gene is preferably in the same genetic locus as the chimaeric DNA sequence of this invention in any plasmid, phasmid or shuttle vector used to transform the Actinomycetale strain. For good expression of the selectable marker gene, it can be driven by the same types of promoter and/or ribosome binding site used in the chimaeric DNA sequence of this invention to obtain good expression of the antigen-encoding gene. However, this is not believed necessary, and the endogenous promoter and RBS of the marker gene or of other promoters, conventionally used with the marker gene, can generally be suitably utilized.

An Actinomycetale strain, particularly a strain of mycobacteria, can be transformed with the chimaeric DNA sequence of this invention, as well as a marker gene, in a conventional manner. In this regard, the strain of Actinomycetale can be transformed by incorporating the chimaeric DNA sequence of this invention into a suitable E. coli/Actinomycetale shuttle vector and then subjecting the vector and the Actinomycetale strain to electroporation. For example, the electroporation procedures of GICQUEL-SANZEY et al (1989) and RANES et al (1990) can be suitably used, as well as the procedures generally described in EP 400,973. Alternatively, the chimaeric DNA sequence of this invention can be incorporated: into a shuttle phasmid and used to transform an Actinomycetale, particularly a mycobacteria, strain in accordance with PCT publication WO 88/06626; into a secretory expression vector and used to transform an Actinomycetale strain in accordance with EP 400,973; into a conjugative plasmid used to transform the Actinomycetale strain in accordance with LAZRAQ et al (1990); or by transposition using a vector for transposon delivery (i.e., with an integrative plasmid, replicative plasmid or phage).

The recombinant immunogenic Actinomycetale strain of this invention can be used in an immunogenic composition, preferably a vaccine, for example, to render a mammal, particularly a human, resistant to a pathogen of the immunizing antigen encoded by the antigen-encoding gene of the chimaeric DNA sequence of this invention. Preferably, the recombinant Actinomycetale strain is a live non-pathogenic strain such as M. bovis BCG or is a strain which has been inactivated, for example by heating it or by treating it with formalin, if necessary. The recombinant Actinomycetale strain can then be mixed with a conventional pharmaceutically acceptable vehicle, such as a physiological saline solution, together with conventional excipients, such as sodium glutamate, carbohydrates, glycerol, amino acids, detergents etc., to form a vaccine. The vaccine can be formulated to contain a final concentration of cell material in a range of 0.2 to 5 mg/ml, preferably 0.5 to 2 mg/ml. After formulation, the vaccine can be placed in a sterile container, which is then sealed and stored at a low temperature (e.g., 4°C), or it can be freeze-dried or fresh frozen.

In order to induce immunity in a human host, one or more doses of the vaccine, preferably just one suitably formulated dose, can be administered in doses each containing about 10^5-10^7, preferably about 10^6, cells. The vaccine can be administered by different routes, for example by intradermal (ID), subcutaneous (SC), percutaneus, oral, spray or aerosol routes.

The Examples, which follow, involve the construction of vectors providing an efficient expression in M. bovis BCG of the HIV-1 gene encoding the Nef protein ("Nef") as an example of an eucaryotic viral gene. The HIV-1 Nef-encoding gene ("nef") is used as a model to study gene expression and induction of a cellular immune response by a recombinant M. bovis BCG vaccine of this invention. Nef is a 27 kD regulatory protein encoded by a single open reading frame which overlaps the 3' long terminal repeat (LTR). Nef is myristilated and localized in the cytoplasm associated with the inner face of the HIV-1 cellular membrane (GUY et al, 1987). Nef is expressed at an early stage of the HIV infection and is involved in the down-regulation of CD4 cell-surface receptors of permissive T4 lymphocytes (GUY et al, 1987; GARCIA and MILLER, 1991). CTL (RIVIERE et al, 1989; CULMANN et al, 1989) has been detected in HIV seropositive donors. It is believed that the recombinant M. bovis BCG vaccine of the Examples, expressing Nef, could induce the destruction of infected cells which express Nef at an early stage of HIV infection, before HIV can release newly synthesized viral particles. This would be a desirable result from a vaccine.

The results of the Examples show that the promoter of the groES/groEL1 operon from S. albus can be used to express the HIV-1 Nef protein in recombinant nef-BCG. The HIV-1 nef gene was cloned on a replicative plasmid derived from pRR3 (Fig. 1) and expressed under the control of the pL promoter of phage lambda and the promoter of the groES/groEL1 operon of Streptomyces albus to accumulate the Nef protein in the BCG cell. The groEs/groELI promoter provided a high level of Nef expression in M. bovis BCG. As a result, a high and specific proliferative response of lymph node

cells was induced in mice inoculated with the immunogenic recombinant mycobacteria in which the promoter of the chimaeric DNA sequence of this invention was the groEs/groEL1 promoter. In this regard, the total amount of Nef protein was estimated to be 1% of the total cellular protein, but no degradation of Nef was observed, and lymph node cells from mice immunized with nef-BCG strongly proliferated in response to purified Nef protein.

The Figures referred to in the Examples are as follows:

Fig. 1. Schematic structure of the "mini" E. coli-mycobacteria shuttle cloning vector pRR3 of Example 1. A thin line shows the 2.58 kb fragment from pAL5000 (RANES et al, 1990). Sequences from pUC19 are shown as a thick line. Origins of replication for E. coli and mycobacteria are shown. This plasmid contains the ApH (3') phosphotransferase-encoding gene from Tn903, conferring resistance to kanamycin ("Kan'), and the ampicillin resistance ("Amp'") gene from pBR322.

Fig. 2. Construction of pWRIP1 and pWRIP2 of Example 1. pTG1166 (GUY et al, 1987) was totally digested with HindIII and partially cut with XhoI. The 1.2 kb XhoI-HindIII fragment, containing the pL promoter from phage Lambda and the complete Nef protein-encoding sequence (nef), was purified from agarose gel. Ends were filled in with Klenow polymerase. The fragment was then inserted in the ScaI site of pRR3 in both orientations to give rise to pWRIP1 and pWRIP2.

Fig. 3. Western blot analyses of recombinant M. smegmatis and M. bovis BCG clones of Example 1:

Fig. 3A: Proteins from M. smegmatis recombinant clones carrying pWRIP1 (lanes 4 and 5) and pWRIP2 (lanes 6 and 7) were extracted and separated in a 10% SDS-polyacrylamide gel. After electroblotting, membranes were reacted with a monoclonal antibody directed to HIV-1 Nef protein. Molecular weight marker appears on lane 1, and positive control containing purified Nef protein (27 kD) is shown on lane 2. Lane 3 contains an extract from non-recombinant M. smegmatis as a negative control. The lower amount of Nef protein observed in lane 6 was attributed to a lower amount of total proteins in this extract.

Fig. 3B: Proteins were extracted from M. bovis BCG recombinant clones carrying pWRIP1 (lanes 3 and 4) and pWRIP2 (lanes 5 and 6). Western blot analysis was performed as described for Fig. 3A. Molecular weight marker and positive control appear on lanes 1 and 2, respectively. Extract of non-recombinant BCG as negative control is shown on lane 7.

Fig. 4. A schematic representation of PCR amplification of Example 2. The groES/groEL1 region with its unique restriction sites is represented as a white box. The primers L1 (5'CCCAGTACTCTAGACCGGCCGGGCTGAGGTTGGCTGGCT3') and L2 (5'CCCCATATGGATCCCTCCCCCTTCGGAGATCACGGGGTTA3'), used for amplification of the promoter region, are represented by arrows, and the amplification product is represented by a black box. The positions of the initiation of transcription and translation are shown. XbaI and BamHI unique sites, introduced with L1 and L2, allowed the cloning of the PCR product into a Blue Script vector to give pWRIP5.

Fig. 5. Construction of pWRIP17 and pWRIP19 of Example 2. pWRIP15, which contains the groES/groEL1 PCR amplified promoter (French application 9011186) and nef gene with the synthetic ribosome binding site from pTG1166, was cut with XbaI and XhoI. The 1.1 kb fragment containing the groES/groEL1 promoter and nef expression cassette was purified from agarose. Ends were filled in with Klenow polymerase and ligated to pRR3 cut with ScaI. Recombinant plasmids, pWRIP17 and pWRIP19, carrying the cassette inserted in both orientations, were isolated from kan^r E. coli cells.

Fig. 6. Western blot analyses of E. coli and M. bovis BCG recombinant clones carrying pWRIP17 and pWRIP19 of Example 2:

Fig. 6A: 10 µg of total proteins from non-recombinant E. coli cells (lane 3) and E. coli transformed with plasmids pWRIP17 (lane 4) and pWRIP19 (lane 5) were extracted and separated with a 10% SDS-polyacrylamide gel. Western blot analysis was performed using a monoclonal anti-Nef antibody. Molecular weight marker and purified Nef protein (0.5 µg) appear on lanes 1 and 2, respectively.

Fig. 6B: 100 µg of total proteins extracted from recombinant M. bovis BCG clones carrying pWRIP17 (lanes 4 and 5) and pWRIP19 (lanes 6 and 7) were analyzed as described for Fig. 6A. Negative control containing proteins from non-recombinant BCG appear on lane 3. Molecular weight marker and purified Nef protein are shown on lanes 1 and 2, respectively.

Fig. 7. Lymph node ("LN") proliferative response of Balb/c immunized mice in Example 3. Balb/c mice were injected s.c. with 10^7 CFU of standard 1173P2 BCG (GHEORGHIU et al, 1983) or recombinant BCG carrying either pWRIP17 or pWRIP19 of Example 2. LN cells

were stimulated with antigens before incorporation of tritiated methyl thymidine (^{3}H)dThd). The responses depicted are the geometrical means of results from three individuals for each animal group. The LN cells from non-immunized mice only responded to concanavaline A.

Materials and Methods

1. Construction of Plasmids

DNA fragments were extracted from agarose gels by using the Gene Clean II kit (Bio 101 Inc., USA) or the Mermaid kit (Bio 101) depending on the length of the fragment to be purified.

Standard procedures were used for DNA ligation, restriction enzyme digestion and transformation in E. coli strains (SAMBROOK et al, 1989).

2. Bacterial Strains and Cultures

E. coli XL1 Blue strain (BULLOCK et al, 1987) was grown at 37°C in L broth medium (MILLER et al, 1972). E. coli TGE901 (Transgene of Strasbourg, France), containing the thermosensitive repressor c1857 of phage lambda, was described by GUY et al, 1987. The transformed E. coli cells were grown at 30°C in L broth, and a two-hour shift at 42°C was performed to induce expression of Nef protein under control of the phage lambda promoter.

M. bovis BCG (Institut Pasteur, Paris, France; GHEORGHIU et al, 1983 and M. smegmatis mc^2 155 (SNAPPER et al, 1990) were transformed with the various recombinant plasmids by electroporation as described by RANES et al (1990).

Two methods of culture were used for BCG recombinant cell preparations. Firstly, the BCG recombinant, as well as a reference BCG strain, were grown in dispersed cultures (GHEORGHIU et al, 1988). BCG clones transformed with the various recombinant plasmids were grown on Löwenstein-Jensen medium containing 10 µg of kanamycin per ml. The kanamycin resistance gene from Tn903 (OKA et al, 1981) was used as a selective marker; it encodes an APH(3') phosphotransferase which is able to phosphorylate kanamycin but has no effect on tobramycin. Therefore, transformant clones were identified by their resistance to kanamycin and sensitivity to tobramycin. Two clones of each recombinant BCG strain were grown in modified Beck-Proskauer medium (GHEORGHIU et al, 1988) complemented with 10 µg of kanamycin per ml. The bacterial content of 10 days old cultures was 10^9 colony forming units (CFU) per ml. Strains expressing the cloned chimaeric DNA sequence of this invention were inoculated into mice.

3. Preparation of Total Cellular Extracts

E. coli strains extracts were prepared from 5 ml cultures grown overnight in L broth. M. smegmatis mc^2 155 strains were grown in Beck-Proskauer medium supplemented with Tween 80 (0.005%) and OADC (Difco), using the same culture conditions as previously described for M. bovis BCG strain. For recombinant cultures containing plasmids derived from pRR3, 25 µg of kanamycin per ml were added.

Cells were centrifuged at 3000 rpm for 10 min. at 4°C, and pellets were washed in TE buffer (10mM Tris, pH 8; 1mM EDTA). Pellets were then resuspended in 0.3 ml of TE buffer and frozen at -20°C for 10 min. Cells were sonicated for 15 sec. periods during 1 min. 1% SDS was then added, and the extract was boiled 3 min. Cell extracts were centrifugated at 11000 rpm for 10 min. at 4°C, and supernatants were collected. When necessary, proteins were concentrated with acetone on ice for 30 min. and resuspended in a small volume of TE buffer. Amounts of proteins contained in the extracts were measured using the BIORAD microprocedure standard assay.

4. Electroporation of Mycobacteria

Exponential cultures of mycobacteria were washed, resuspended (10^8 to 10^9 bacteria/ml) in 10% sucrose, 8mM HEPES, pH 7.4, and 1mM $MgCl_2$, chilled on ice for 30 min. and electroporated at 6.25 Kv/cm, 25µF using the BIORAD Gene Pulser. Cultures were then diluted 10 times in Middlebrook 7H9 (Difco), incubated for about 12 hours at 37°C and plated on Middlebrook 7H10 (Difco) containing 10 to 40 µg/ml kanamycin. M. smegmatis transformants appeared after 5 days, and BCG transformants appeared after three weeks.

5. Western Blot Analysis

10 µg E. coli extracts or 100 µg mycobacterial extracts of soluble proteins were separated with SDS-polyacrylamide 10% gels (LAEMMLI, 1970). 10 µg of molecular weight marker proteins (Bethesda Research Laboratories) and 1 µg of purified recombinant E. coli Nef protein (Transgène), as positive control, were loaded on the gels.

After separation, polyacrylamide gels were electroblotted onto Immobilon membranes (Millipore, Corp.) using a Semiphor TE70 apparatus (Hoeffer Scientific Instruments). Non-specific binding was blocked by incubating the membranes in PBS (10 mM sodium phosphate; 150mM NaCl, pH 7) at room temperature (25°C) with 3% non-fat milk powder and 0.1% Tween 20, for 15 min. at

room temperature. The proteins were then reacted over night with a 1:1000 diluted monoclonal antibody directed to Nef protein (NEN from Dupont). The membranes were washed three times for 10 min. each time with PBS and 0.1% Tween 20 and reacted with phosphatase alkaline-conjugated goat anti-mouse IgG (BIOSYS) at a 1:1000 dilution for 1 h at room temperature. After three times washing for 10 min each time with PBS and 0.1% Tween 20, the blots were reacted as described in Protoblot procedure (Promega).

6. Immunization of Mice

Female Balb/c mice (8 weeks) were immunized subcutanously (sc) at the base of the tail with incomplete Freund adjuvant (IFA from DIFCO) containing 10^7 colony forming units (CFU) of recombinant BCG strains or standard M. bovis BCG 1173 P2. After 14 days, draining inguinal lymph nodes were removed, and cell suspensions, pooled from three mice, were prepared (ANDERSEN et al, 1991). A single cell suspension of LN was prepared in RPMI 1640 (GIBCO) containing 2mM L-glutamine, 50µg/ml gentamycin, $5X10^{-5}$M 2-mercaptoethanol, and 10% fetal calf serum (FCS). T-cells were cultivated at a concentration of $4x10^5$ cells/well in 96-well flat bottom culture plates in the presence of the appropriate antigen. Concentration of the antigens added in cell cultures was on the basis of dose stimulation response as follows: Nef, 1 and 10 µg/ml; APH(3') 0.1 and 1 µg/ml; Protein Purified Derivative (PPD), 10µg/ml; and Concanavalin A (ConA from Sigma), 2.5µg/ml. Some cells were left unstimulated. Each test was performed in triplicate. Cultures were incubated for 5 days at 37°C in humidified air with 7% CO_2, the last 22 h in the presence of tritiated methyl thymidine (^{3}H)dThd (1mCi/ml). The cells were harvested on glass fiber filters with Automash 2000 Dynatech (Bioblock, France), and the incorporated radioactivity was measured in liquid scintillation counter (Beckman). The results are expressed as mean counts per minute (cpm) minus background.

Example 1. Expression of HIV-1 Nef-encoding gene Under the Control of the lambda Phage pL Promoter

pRR3 (RANES et al, 1990) was chosen as a vector for the cloning of the nef gene in M. bovis BCG. Its structure is shown in Fig. 1. It contains a pAL5000 2.58 kb fragment essential for replication and maintenance in mycobacteria, the Kan^r gene from Tn903, and a pUC19 fragment harbouring an E. coli origin of replication. This plasmid was shown to be stable in M. bovis BCG when the bacteria are grown in in vitro laxenic cultures or in mice.

The Nef-encoding gene was previously cloned on the pTG1166 plasmid and expressed in E. coli under the control of the pL promoter of phage lambda (GUY et al, 1987). Since several gram-negative bacterial genes, such as antibiotic resistances, are expressed in mycobacteria under control of their own regulation signals, nef expression has also been examined in M. smegmatis and M. bovis BCG under pL control. The nef gene, containing a XhoI-HindIII fragment of pTG1166, was inserted into pRR3 at the ScaI site giving rise to pWRIP1 and pWRIP2, according to the orientation of the fragment (Fig. 2). These plasmids were transferred into M. smegmatis mc^2-155 strains by electroporation. M. Smegmatis kanamycin resistant (Kan^r) transformant clones were examined for nef expression by western blot analyses. As shown in Fig. 3A, with both orientations of the nef containing fragment in pRR3, expression of nef was observed, suggesting that the pL promoter is active in mycobacteria.

When pWRIP1 and pWRIP2 were transferred into M. bovis BCG by electroporation, expression of nef was also detected, albeit to a lesser extent (Fig. 3B).

Example 2. Expression of HIV-1 Nef-encoding gene under the control of s. albus groES/groEL1 promoter

In order to obtain a higher expression of nef in mycobacteria, this gene was cloned under the control of a stress-responsive promoter from Streptomyces (S. albus), a genus closely related to Mycobacteria. The aim was to induce an immune response to the Nef protein after inoculation of mice with M. bovis BCG recombinants harboring the nef gene. It was considered essential to clone nef under the control of a promoter which would be active when M. bovis replicates within macrophages so that the Nef antigen(s) would be expressed in the macrophages. The nef gene was therefore cloned under the control of a Streptomyces heat-shock promoter.

In order to examine the transcription of the S. albus groEL2 and groES/groEL1 promoters (French application 9011186; Appendix A) in mycobacteria, they were first fused to the kanamycin resistance gene from Tn5 encoding APH(3') phosphotransferase. High levels of kanamycin resistance (up to 5 mg/ml) were obtained in both M. smegmatis and M. bovis after electroporation with the recombinant plasmid constructions. However, Western blot analysis revealed that the groES/groEL1 promoter was more efficient.

A DNA fragment containing the promoter and the translation initiation control region of the

groES/groEL1 region was synthesized by PCR using primers L1 and L2 containing respectively single XbaI and BamHI restriction sites. The fragment was then inserted into a Blue Script vector (Pharmacia, Sweden) to give pWRIP5 (Fig. 4). The HIV-1 BamHI-HindIII nef-containing fragment was then subcloned into pWRIP5 between BamHI and HindIII sites. The resulting plasmid pWRIP5F contained the entire nef coding sequence following the ATG start codon of the groEs gene. The groES promoter and nef coding sequence XbaI-HindIII fragment from pWRIP5F was inserted into the ScaI site of pRR3 in both orientations. The resulting plasmids pWRIP7 and pWRIP9 were electroporated into M. smegmatis and M. bovis BCG. Neither M. smegmatis nor M. bovis BCG transformants expressed Nef. This result could be attributed to the 8-base pair changes introduced inside the groES/groEL1 translation controlling region during the cloning.

Since the nef gene was expressed in mycobacteria under the control of pL promoter associated with a synthetic ribosome binding site, the ClaI-HindIII fragment of pTG1166 containing this element was inserted into pWRIP5 (Fig. 5). This resulted in plasmid pWRIP15. The XbaI-XhoI fragment from pWRIP15 was then subcloned into the ScaI site of pRR3 in both orientations to give pWRIP17 and pWRIP19. On these two plasmids, the nef gene is under the control of the S. albus groES/groEL1 promoter and a synthetic ribosome binding site. pWRIP17 in E. coli was deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) of Institut Pasteur under accession no. I-1109 on June 6, 1991.

A high level of Nef synthesis was observed in E. coli cells transformed with pWRIP17. However, no expression of nef gene was detected in E. coli cells transformed with pWRIP19 (Fig. 6A). This result could be due to the ampicillin resistance (Amp') gene, the promoter of which was cloned in an opposite direction to nef and which might downregulate its expression. A high level of Nef synthesis was observed in M. smegmatis and in M. bovis BCG bacteria transformed with pWRIP17 and pWRIP19 (Figs. 6A and 6B). The Nef protein was estimated to be about 1% of the total cellular protein. Nef was shown to be expressed in M. bovis BCG cultured in various growth conditions: in dispersed grown cultures and in Sauton cultures. In addition, expression of the APH(3') phosphotransferase was also detected by Western blot analysis in the different M. bovis BCG culture preparations.

Example 3. Determining proliferative responses to Nef and to APH3' antigens in mice immunized with recombinant M. bovis BCG

Balb/c mice were immunized by subcutaneous inoculation with recombinant M. bovis BCG harboring plasmids pWRIP17 and pWRIP19 from Example 2. Cellular responses were investigated by examining the proliferative response of cells isolated from lymph nodes (LN). These cells were cultured in vitro and stimulated with purified Nef or APH(3'). As shown in Fig. 7, LN cells isolated from mice immunized with Nef- and APH(3')-recombinant M. bovis BCG strongly proliferated to purified Nef or APH(3'). This proliferation was highly specific since mice immunized with non-recombinant BCG or with incomplete Freund adjuvant alone did not respond to these heterologous proteins.

In contrast, the proliferation to PPD was comparable in all mice inoculated with non-transformed M. bovis BCG and recombinant strains (Fig. 7). No significant proliferation was observed in LN cells isolated from non-immunized mice. The non-specific proliferative responses of LN cells to concanavaline A (ConA) was the same in all animals.

These results show that, after inoculation in mice, expression of Nef and APH(3') in recombinant BCG induces the specific proliferation of LN cells from immunized mice.

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Claims

1. An immunogenic strain of Actinomycetale, particularly mycobacteria, transformed with the following, operably linked, chimaeric DNA sequence, comprising an antigen-encoding gene that: a) is foreign to the Actinomycetale strain to be transformed; b) encodes a desired antigen, preferably an immunizing antigen; c) is

under the control of a promoter foreign to the Actinomycetale strain to be transformed, preferably a promoter from another strain of Actinomycetale, especially a promoter from another species of Actinomycetale; and d) is preferably associated with a ribosome binding site foreign to the Actinomycetale strain to be transformed, particularly foreign to Actinomycetale.

2. The transformed strain of claim 1 wherein the promoter is a Streptomyces promoter, especially a stress-responsive (e.g., heat-shock) promoter, particularly wherein the transformed strain is a mycobacteria strain.

3. The transformed strain of claim 1 or 2 wherein the ribosome binding site is a synthetic ribosome binding site, preferably having the sequence:

5'ATCGATAACAGAGGAACAGATCT3'.

4. The chimaeric DNA sequence of anyone of claims 1-3, especially wherein the antigen-encoding gene codes for an HIV-1 protein, particularly the Nef protein, or an antigenic fragment or an epitope thereof, quite particularly wherein the antigen-encoding gene is under the control of the S. albus groES/groEL1 promoter.

5. A system, such as a plasmid, capable of transforming a strain of Actinomycetale, particularly a strain of mycobacteria, comprising the chimaeric DNA sequence of claim 4, particularly pWRIP17.

6. An immunogenic composition, particularly a vaccine, comprising the transformed strain of Actinomycetale, particularly mycobacteria, quite particularly BCG, of claim 1, and optionally a suitable carrier.

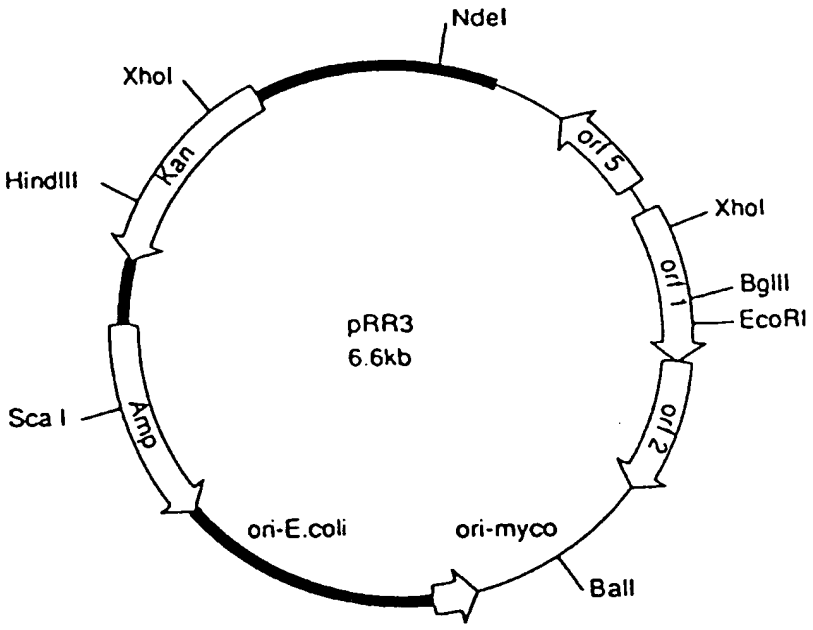
7. A method of treating, preferably immunizing, a mammalian host, particularly a human, comprising the step of administering to the host the composition of claim 6.

8. A process for producing a desired antigen, preferably an immunizing antigen, comprising the step of culturing the transformed strain of anyone of claims 1-3.

9. A process for producing the transformed strain of anyone of claims 1-3 comprising the step of electroporating the Actinomycetale strain with the system of claim 5.

10. A proces of diagnosing the presence of, or reacting with, an antibody in a sample, comprising the step of contacting the transformed strain of anyone of claims 1-3 with the sample.

FIG. 1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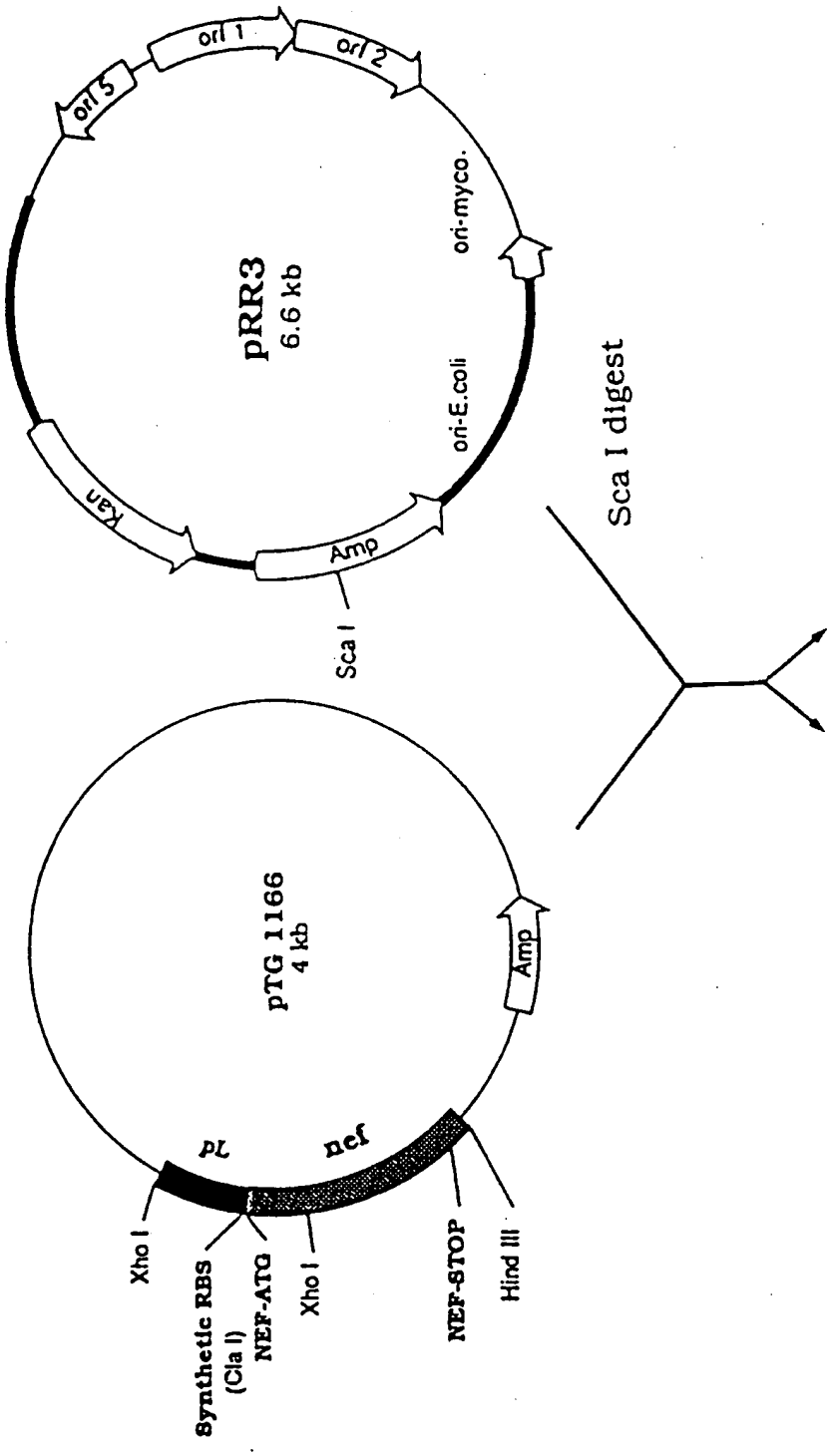
ori-myco
BalI

FIG. 2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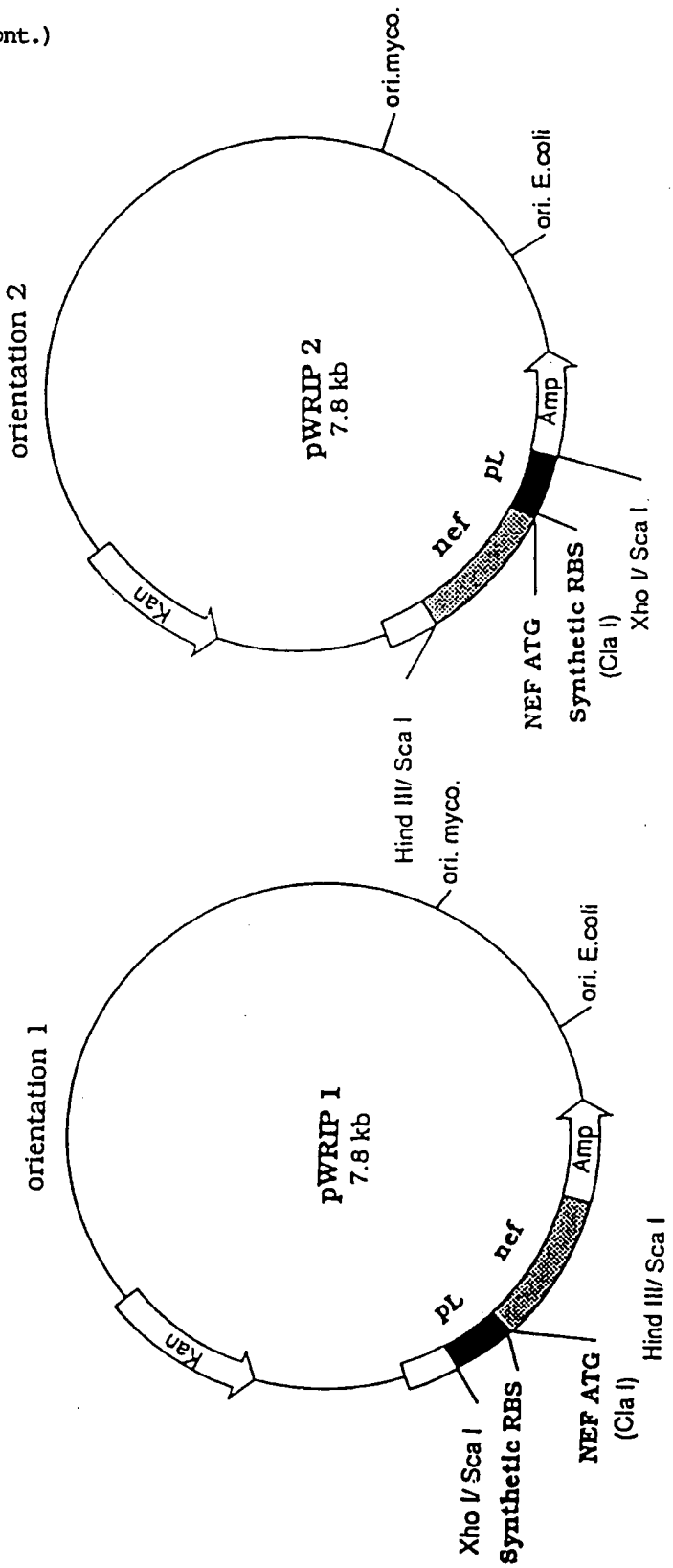
Hind III

FIG. 2 (cont.)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Xho I/ Sca I
Hind III/ Sca I
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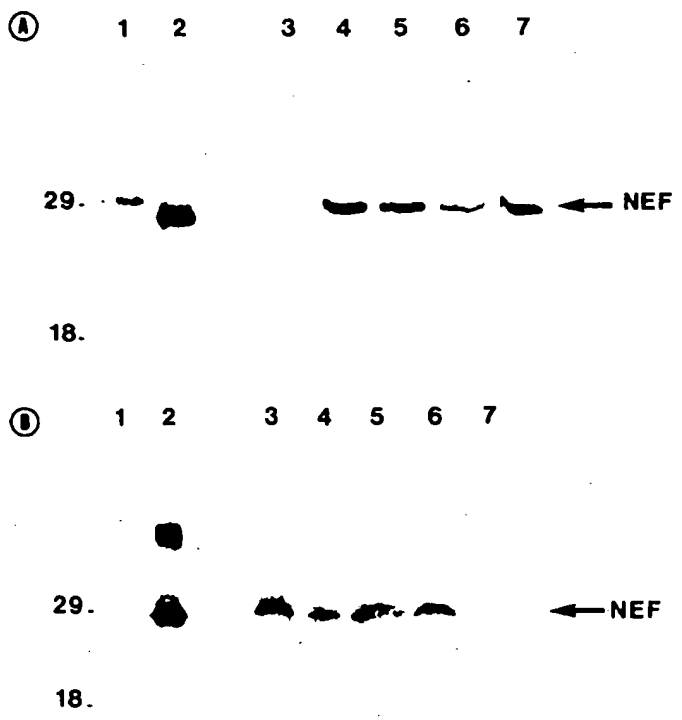
Hind III/ Sca I

FIG. 3



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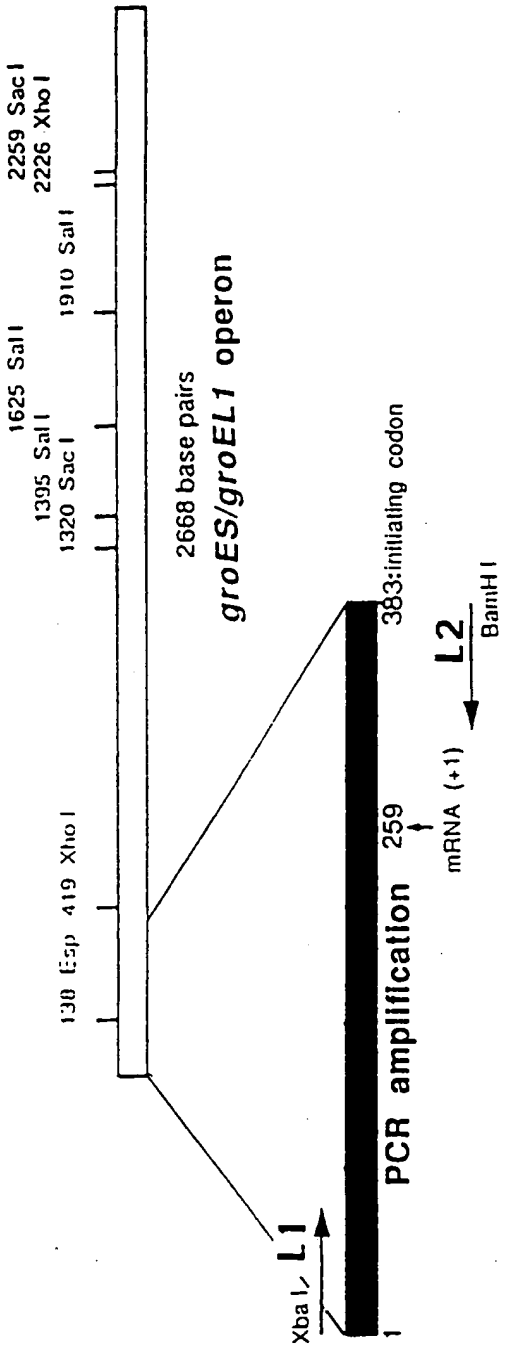
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BamH I
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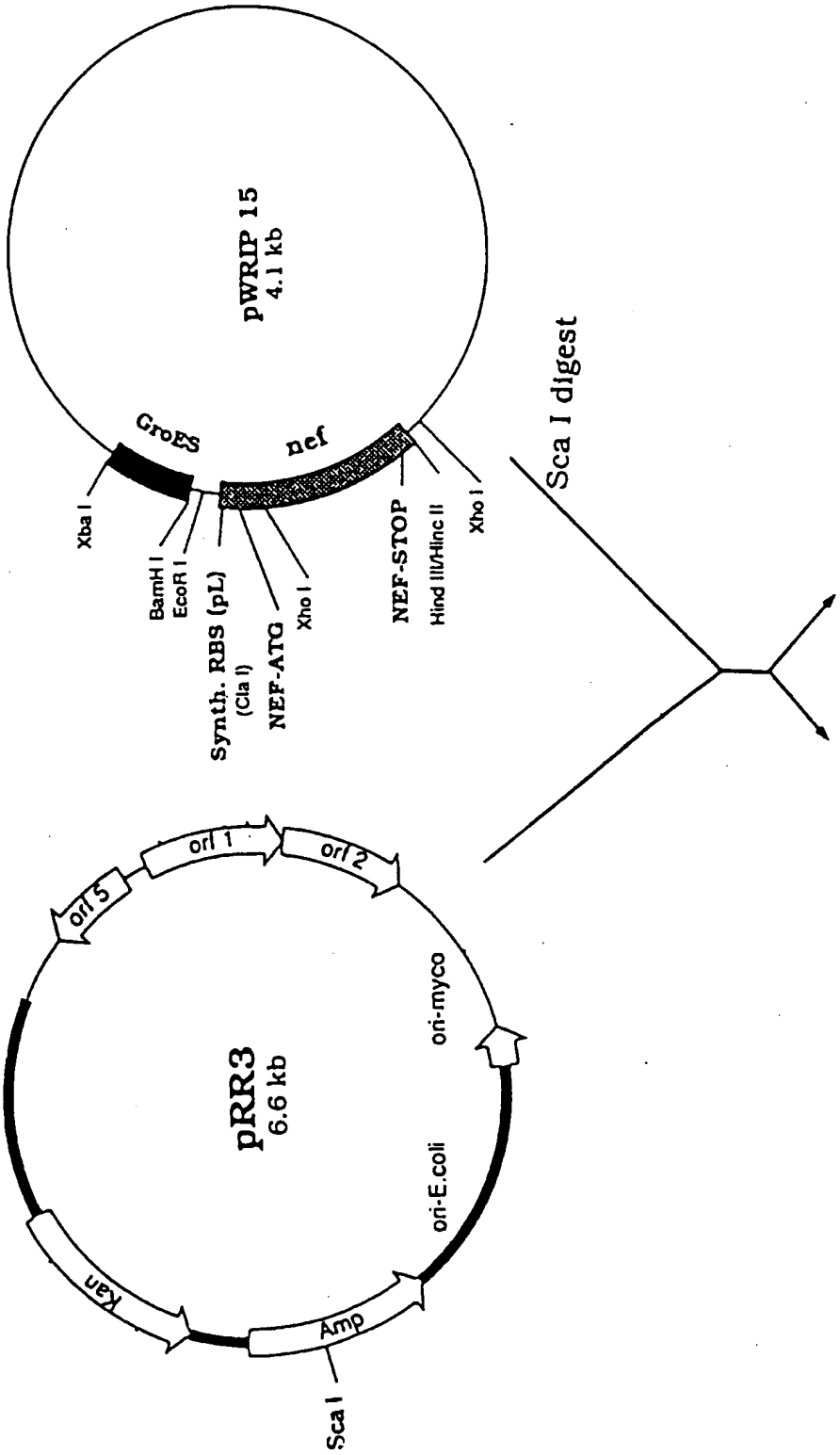
259
PCR amplification
Xba I
L1
1

FIG. 5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ori-E.coli
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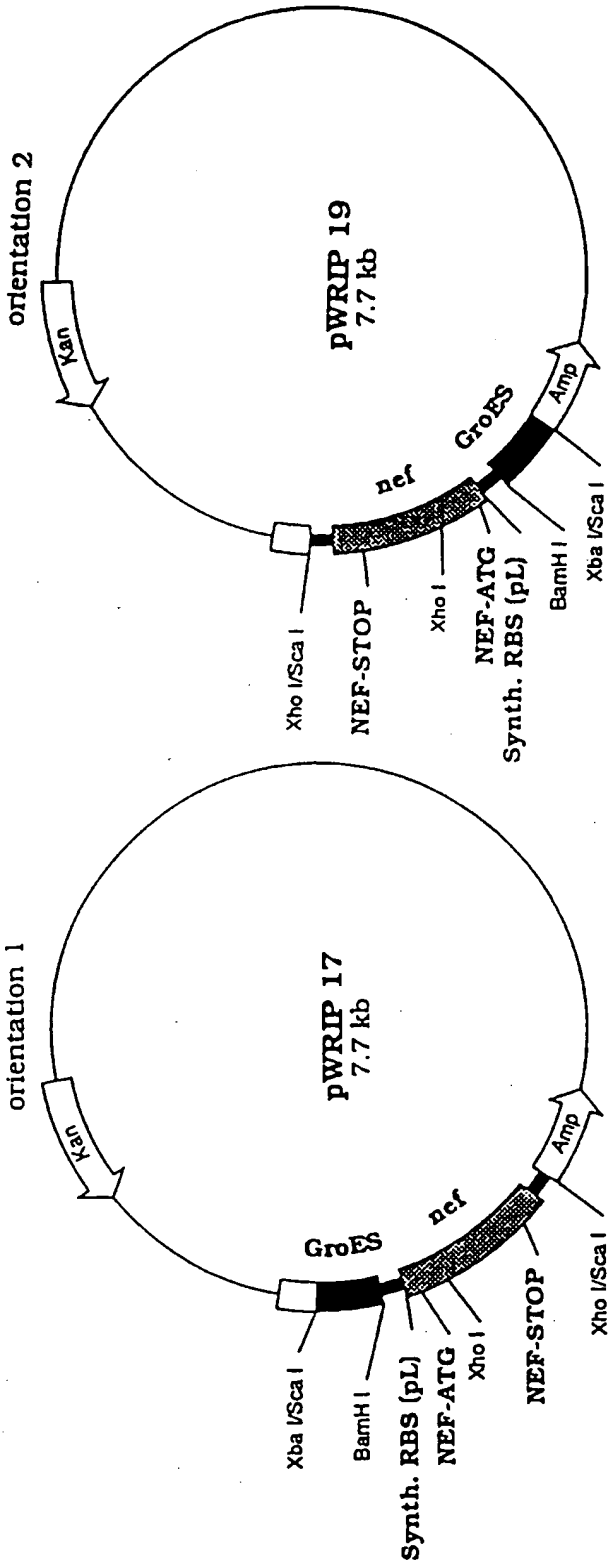
Sca I

FIG. 5 (cont.)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Amp
GroES
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Xho I/Sca I

FIG. 6

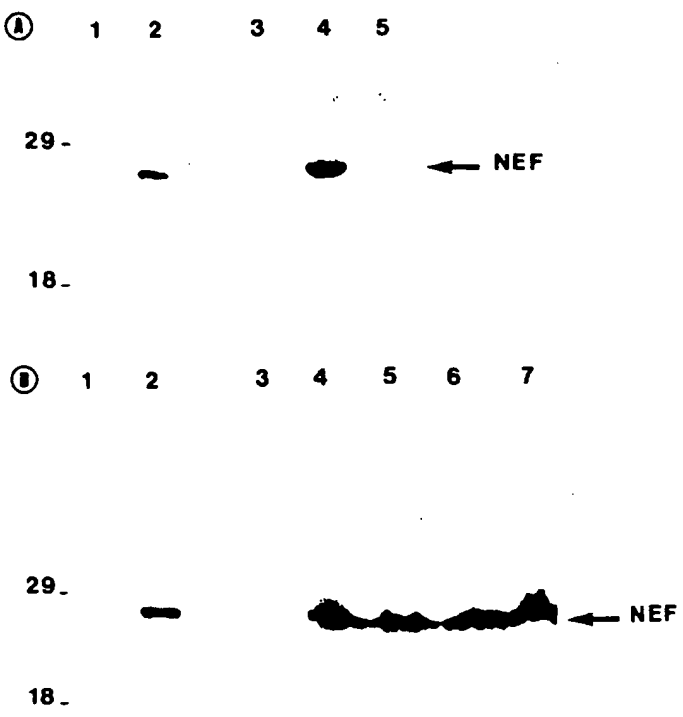
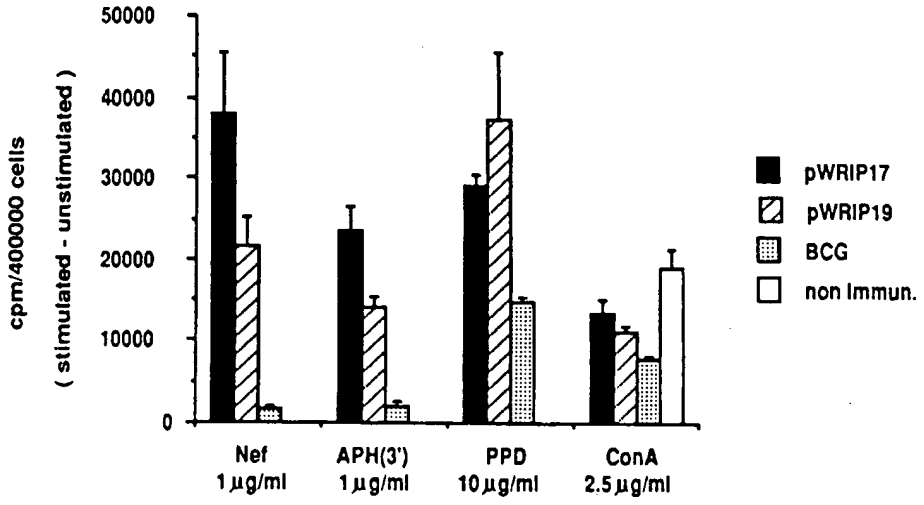


FIG. 7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20000
10000
0
cpm/400000 cells
(stimulated - unstimulated)
Nef
1 µg/ml
APH(3')
1 µg/ml
PPD
10 µg/ml
ConA
2.5 µg/ml
pWRIP17
pWRIP19
BCG
non Immun.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 40 1601

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	WO-A-9 010 701 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) * whole document; in particular page 6, lines 1-10 *	1,6,8,9	C 12 N 15/74 A 61 K 39/04
X	RES. MICROBIOL. vol. 141, nos. 7,8, 1990, pages 931-939, Paris, FR; R.G. BARLETIA et al.: "Recombinant BCG as a candidate oral vaccine vector" * whole article *	1,6-10	
D,X	EP-A-0 400 973 (AJINOMOTO CO., INC.) * whole document *	1,6-10	
D,Y		2-5	
Y	WO-A-9 015 873 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) * whole document *	2-5	
A		1,6-10	
A	WO-A-9 000 594 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH et al.) * whole document *	1,6-10	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N 15/76 C 12 N 15/74
A	WO-A-9 104 051 (MEDIMMUNE, INC.) * page 7, example 1 *	4,6-10	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	15-01-1992	JULIA P.

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L : document cited for other reasons

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